Brief Description of the Drawings

Fig. **1** illustrates a design scheme for oligonucleotides, in which a linker 1 contains a portion that is specific for anchor 1 and another portion (a probe) that is specific for target mRNA 1, and in which a labeled detection probe 1 is specific for a sequence of target mRNA 1 which is different from the sequence of the target-specific portion of the linker.

Fig. **2** illustrates a surface which comprises 15 test regions, each of which comprises an array of six anchor oligonucleotides.

Fig. **3** illustrates the design of a linker for a receptor binding assay, in which the anchor-specific portion of the linker is associated with the probe portion (the receptor protein) via biotin and streptavidin molecules, and in which a ligand specific for the receptor is labeled with a fluorescent labeling molecule. B: Biotin. SA: Streptavidin. Rec: Receptor protein. Ligand: a natural or synthetic ligand for the receptor. *: a fluorescent labeling molecule attached to the Ligand.

Fig. **4** illustrates a surface which comprises 21 test regions, each of which is further subdivided into 16 subregions (indentations, dimples).

Figs. **5a, 5b** and **5c** illustrate three pieces from which a surface such as that shown in Fig. 4 can be assembled. Fig. 5a represents a well separator; Fig. 5b represents a subdivider; and Fig. 5c represents a base.

Fig. **6** represents two test regions, each of which comprises a linear array of probes (or anchors) which are in a "bar-code"- like formation.

Fig. **7** schematically represents a test region comprising 3 anchors (A, B and C), each of which is present in multiple copies (a "group"). The location of each group of anchors is termed a "locus."

Fig. **8** illustrates an assay in which cDNA(s) generated by specific reverse transcriptase are assayed on MAPS plates.

Fig. **9** illustrates an assay which uses a nuclease protection procedure (NPA-MAPS assay). Sample RNA is prepared from cells or from tissue and is represented as thin wavy lines. To the RNA sample is added a group of polynucleotide protection fragments,

portrayed as thick, dark and light lines. The dark sections of the protection fragments represent segments that are complementary to specific RNA targets and hybridize to those targets. The light sections represent overhanging portions: sequences contiguous with the complementary sequence but not complementary to target. The protection fragments are added in excess. Following hybridization of all available target to the protection fragments, the samples are treated with an appropriate cocktail of nucleases and with chemical treatments that destroy unwanted non-hybridized RNA and non-hybridized polynucleotide. For example, S1 nuclease can destroy any single stranded DNA present. Hence, excess protection fragment is hydrolyzed as is the overhanging non-hybridized portion of bound protection fragment. RNA can be hydrolyzed by addition of ribonucleases including ribonuclease H and or by heating samples in base. Remaining is a collection of cleaved protection fragments that reflect how much of each target RNA had been present in the sample. The remaining protection fragments are measured by a MAPS hybridization assay.

Fig. **10** illustrates hybridization specificity in a MAPS assay.

Fig. **11** illustrates binding kinetics of an anchor to a linker.

Fig. **12** illustrates a MAPS assay of two oligonucleotide targets.

Fig. **13** illustrates the quantification of a sensitivity shift.

Fig. **14** illustrates melting temperature determinations for four oligonucleotide linker/anchor combinations.

Fig. **15** illustrates an mRNA assay by NPA-MAPS.

Fig. **16** illustrates a dilution curve with NPA-MAPS.

Fig. **17** illustrates an assay to detect peptides containing phosphotyrosine residues.

Fig. **18** illustrates the first step in an assay to map ESTs: assembling linkers corresponding to each of the ESTs to be mapped on arrays of generic anchors on a MAPS plate. To the surface of each of 16 wells of a microplate are attached linkers comprising 16 different oligonucleotide probes, arranged in a 4x4 matrix. The first locus has oligo 1, which is complementary to a portion of the first EST sequence, and so on for the 16 ESTs to be tested.

cDNAs or mRNAs generated from the genes from which the ESTs were obtained are

added to all 16 wells and allowed to hybridize under appropriate conditions. Hence, any cDNA or mRNA that contains one of the 16 EST sequences will be assembled at the locus where its complementary probe was placed.

Fig. **19** illustrates a subsequent step in an assay to map ESTs: adding detector oligonucleotides to the MAPS plate. Each well of the plate receives a detector oligonucleotide which corresponds to one of the ESTs to be mapped. Each detector oligonucleotide is an oligonucleotide coupled to a molecule used for detection, *e.g.*, fluorescein if fluorescence imaging is to be the method of detection. Each detector oligonucleotide is complementary to one of the ESTs, but different from the EST-specific probe, so that a probe and a detector oligonucleotide which are complementary to a single EST can both bind at the same time.

After washing, a single detector oligonucleotide is added to each well, as numbered in the figure. That is, the detector oligonucleotide with sequences complementary to the first EST is added to the first well, and so on.

Fig. **20a** and **b** illustrate the results of the assay to map ESTs shown in Figs. 18 and 19. After hybridization of detector oligonucleotides and washing with appropriate conditions of stringency, the 16 wells of the microplate are imaged with a CCD-based fluorescence imager, for example. Fig. 20a shows stylized results. It is expected that each EST-specific detector oligonucleotide should label the mRNA or cDNA held down by the corresponding EST-specific probe. For example, probe 5 assembles the cDNA or mRNA containing the fifth EST sequence at that locus, so the fifth detector oligonucleotide should also hybridize to the cDNA or mRNA at the same locus. This is the case for these stylized data, with each detection oligonucleotide labeling the matching probe. In addition, the first three detector oligonucleotides each label cDNA or mRNA held down by the first three probes, showing that these sequences lie along the same gene. Similarly, the last five ESTs appear to be linked. The linkage assigned from these data are presented graphically in Fig. 20b.

Fig. **21** illustrates the relationships of the probes, detector oligonucleotides and ESTs #1, 2 and 6 shown in Figures 18-20.

Fig. 22 illustrates a high throughput assay.

Fig. 23 illustrates a method to prepare an amplified target.

Fig. 24 illustrates an assay with detection linkers and reporter agents.

Fig. 25 illustrates a use of multiple flours.

EXAMPLES

Example 1 Hybridization Specificity (see Figure 10)

A generic MAPS plate was produced by using an inkjet dispenser, the Pixus system (Cartesian Technologies, Inc., Irvine, CA) to form an identical grid of DNA within each well of a microtiter plate. All oligonucleotides were purchased from Biosource International (Camarillo, CA). For this plate, seven different oligonucleotide anchors were dispensed within each well in the pattern shown as the Key (left side of the figure). Each oligonucleotide was dispensed as a 10 nanoliter droplet to two spots, from a 2 uM solution containing 500 mM sodium phosphate pH 8.5 and 1 mM EDTA to the wells of a DNA Bind plate (Corning Costar), and allowed to dry. After attachment, wells were blocked with 50 mM Tris pH 8, and then oligonucleotide that had not covalently attached to the surface was washed away with 0.1% SDS in 5x SSP buffer.

To the washed plate fluorescently labeled linker oligonucleotides were added and allowed to hybridize in 6x SSPE with 0.1% Triton X-100 at room temperature for thirty minutes. This is a preferred protocol for attachment of linkers. The linker oligonucleotides were cy5-derivatized during synthesis, and were complementary in 25 base-pair segments to specific anchoring oligonucleotides. The sequences of the seven anchors and linkers were as follows (all shown 5' to 3'):

#1Anchor*: SEQ ID:1
TCCACGTGAGGACCGGACGGCGTCC

Linker** SEQ ID:2
GTCGTTTCCATCTTTGCAGTCATAGGATACTGAGTGGACGCCGTCCGGTCCTCACGTG
GA

RNA mimic(mouse C-jun): SEQ ID:3
CTATGACTGCAAAGATGGAAACGACGATACTGAGTTGGACCTAACATTCGATCTCAT

TCA
Detector Oligonucleotide*** SEQ ID:4
TGAATGAGATCGAATGTTAGGTCCA

#2 Anchor*: SEQ ID:5
CACTACGGCTGAGCACGTGCGCTGC
Linker** SEQ ID:6
CTAGGCTGAAGTGTGGCTGGAGTCTGCAGCGCACGTGCTCAGCCGTAGTG
RNA mimic (mouse MIP-2): SEQ ID:7
AGACTCCAGCCACACTTCAGCCTAGGATACTGAGTCTGAACAAAGGCAAGGCTAACT
GAC
Detector Oligonucloeotide*** SEQ ID:8
GTCAGTTAGCCTTGCCTTTGTTCAG

#3 Anchor*: SEQ ID:9
GTCAGTTAGCCTTGCCTTTGTTCAG
Linker** SEQ ID:10
ACCATGTAGTTGAGGTCAATGAAGGGCGCTCCCACAACGCTCGACCGGCG
RNA mimic (mouse GAPDH): SEQ ID:11
CCTTCATTGACCTCAACTACATGGTGATACTGAGTGGAGAAACCTGCCAAGTATGAT
GAC
Detector Oligonucloeotide*** SEQ ID:12
GTCATCATACTTGGCAGGTTTCTCC

#4 Anchor*: SEQ ID:13
GAACCGCTCGCGTGTTCTACAGCCA
Linker** SEQ ID:14
CTACCGAGCAAACTGGAAATGAAATTGGCTGTAGAACACGCGAGCGGTTC
RNA mimic (mouse L32 protein): SEQ ID:15
ATTTCATTTCCAGTTTGCTCGGTAGGATACTGAGTGAGTCACCAATCCCAACGCCAGG
CT
Detector Oligonucloeotide*** SEQ ID:16
AGCCTGGCGTTGGGATTGGTGACTC

#5 Anchor*: SEQ ID:17
CTCGTTCCGCGTCCGTGGCTGCCAG
Linker** SEQ ID:18
CTGGCAGCCACGGACGCGGAACGAG
#6 Anchor*: SEQ ID:19
CGGTCGGCATGGTACCACAGTCCGC
Linker** SEQ ID:20
GCGGACTGTGGTACCATGCCGACCG

#7 Anchor*: SEQ ID:21
GCGCGCCGCGTTATGCATCTCTTCG
Linker** SEQ ID:22
CGAAGAGATGCATAACGCGGCGCCG

*Anchors were synthesized with C12 spacer with amide at the 5' end
**Linkers were synthesized with Cy5 attached at the 5' end
***Detector Oligonucleotides were synthesized with biotin attached at the 5' end

To each well either one linker or a mixture of linkers (as indicated in the figure) was added in bulk. (To the well marked "all" was added a mixture of all seven linkers.) Following incubation and washing in 5x SSP 3 times, the fluorescence picture shown on the right portion of the figure was taken with a Tundra imager (IRI, St. Catherines, Ontario). As can be seen, the linkers self-assembled to the surface, by specifically associating with their complementary anchors.

This process is repeated except that eight different anchors are dispersed in each well and linkers subsequently preferentially associated therewith. The entire process is repeated with 36, 64 etc. different anchors in each well of a 24, 96, 384, 864 or 1536 well plate.

Example 2 Binding Kinetics (see Figure 11)

The rate of hybridization of Cy5-derivatized linker number 1 to its complementary attached anchor is shown, for different concentrations of linker. The generic MAPS plate was prepared as for figure 1, except anchor 1 was attached at four spots per well. Incubations were done at room temperature in 5x SSP with 0.1% tween-20, wells were washed 3 times with 5x SSP, and bound fluorescence was measured. A fluorescence picture of the plate was taken with the Tundra, and background was subtracted and the integrated intensity of each spot within each well was calculated with Tundra software. Plotted is the average and standard deviation for the integrated intensity for the four spots within each of two duplicate wells.

Example 3 Fluorescent Linker.

A generic MAPS plate is produced with one anchoring oligonucleotide spotted to either 1 spot per well (top two rows), 4 spots per well (next four rows) or 16 spots per well (lower two rows), according to the methods discussed above. To each well complementary, fluorescently labeled, linker is attached by the preferred protocol described in Example 1. Following washing the fluorescence picture of the plate is taken with the Tundra. The amount of fluorescence at each spot reports how much functional linker is available to hybridize to target. The amount of signal detected at repeated spots is highly reproducible.

Example 4 Binding Curves.

To the plate prepared as described in Example 3, is added different concentrations of a target oligonucleotide. The linker that has been associated contains a 25-mer sequence complementary to a portion of the target. The target is added in 5x SSC with 0.05% SDS in a total volume of either 30 or 100 microliters, and the plate is covered and incubated at 50° C overnight. Following hybridization of the target to the attached linker, the target is visualized by a preferred protocol using chemiluminescence. A biotinylated detector oligonucleotide, containing a 25-mer sequence complementary to a separate portion of the target (not to the same portion complementary to linker) is added at 30 nM. Biotinylated detector can be added for 30 minutes after washing away excess unattached target, or it can be added along with target for the length of the overnight hybridization. Following attachment of detector, the surface is washed twice with 5x SSC, once with 1x SSP containing 0.1% Tween-20 and 1% PEG (SSPTP), and a 1:50,000 dilution of 250 ug/ml Horse Radish Peroxidase conjugated to Streptavidin (HRP:SA, from Pierce, Rockford, Ill.) is added for 5 hours in SSPTP at room temperature. Wells are washed four times with SSPTP, and washed once and then incubated with Super Signal Ultra reagent (Pierce). After a few minutes, pictures of luminescence are collected with the Tundra imager, *e.g.*, the picture can accumulate within the CCD array for five minutes. Low levels of target can be visualized in some wells at a target concentration of as little as $\sim 5 \times 10^{-13}$ M; the amount of signal generally becomes saturated at a target concentration of $\sim 10^{-10}$ M. The amount of

signal detected at repeated spots is highly reproducible.

Example 5 Assay of Two Oligonucleotides (see Figure 12)

A binding curve demonstrating a MAPS hybridization assay using the preferred protocol discussed above for two different target oligonucleotides is shown. A generic MAPS plate was prepared with four different anchoring oligonucleotides each spotted four times within each well. For the second and fourth anchor, complementary linker oligonucleotides were self-assembled onto the surface as described. Two targets were added at the concentrations shown in 40 microliters to each well as described, and incubated at 50° C overnight. The amount of each target attached was visualized by attaching biotinylated detection oligonucleotide specific for each target followed by HRP:SA and chemiluminescence imaging as described. In the lower panel the intensity of the image is quantified. Software that is part of the Tundra Imager package was used to scan the intensity of the images along lines between the arrows shown in the upper panel. At the lowest concentration of target, 1.1 pM, the scanned images show well-defined gaussian peaks at each spot, while there are no discernable background peaks seen in the left-most sample, at 0 concentration of target.

Example 6 Sensitivity Shifting (see Figure 13)

A MAPS hybridization assay can be used for measuring the concentration of a set of oligonucleotides, by binding them to a surface and labeling them. This works well for those oligonucleotides which are at modest or low concentration. Two samples can be distinguished in such a case because if one sample contains more oligonucleotide, more will bind. On the other hand, if the concentration of targeted oligonucleotide is saturating for the surface (*i.e.* if it is high enough to occupy all binding sites), then if the concentration goes up no more can bind, so the amount cannot be measured. However, the binding curve of a target can be shifted by adding unlabeled competing ligand.

Binding data are obtained for four different oligonucleotide targets, all of which saturate the surface (*i.e.* reach maximal binding) at roughly 3 nM. By adding unlabeled

competitive targets to all wells, the binding of labeled oligonucleotide is shifted, so that less binds at the lower concentration, and the level at which saturation occurs is moved up. One can add competitive oligonucleotides for, say, targets 1 and 3 but not 2 and 4. This shifts the sensitivity of the assay only for targets 1 and 3. In this way oligonucleotide targets of widely different concentrations can be measured within one assay well, if the relative amount of oligonucleotide expected is known.

The data can be quantified as explained above for the binding of one of the oligonucleotide targets. Figure 13 shows quantitatively that including competitive oligonucleotide in the assay shifts the binding curve used to assay for this target to higher concentrations.

Example 7 Melting Temperature of Four Probes (see Figure 14)

The amount of four different fluorescent labeled linker oligonucleotides specifically hybridized to anchor oligonucleotides by the MAPS assay is plotted as the temperature is raised. The four oligonucleotides were first allowed to hybridize at 50° C for 1 hour at 300 nM. Then the wells were washed with SSC without probes, and the amount bound was measured as above by fluorescence (50° C point). Then the surface was incubated at 55° C for 30 minutes and the fluorescence bound measured, and so on for all temperatures presented.

Example 8 Detection Methods

Two detection methods can be compared directly. To a MAPS plate with four oligonucleotide anchors attached, each at four spots per well, are added two oligonucleotides to each well, with both including a covalently attached cy5 moiety or both containing a biotin group. The epi-fluorescence measurement is performed as described for viewing and measurement of the fluorescent linker. The chemiluminescence measurements are performed as described for the MAPS assay using subsequent addition of HRP:SA and a chemiluminescence substrate. The signals generated are roughly of the same magnitude. However, for the geometry of the microplates, which contain walls separating each well, and

occasional bubbles of liquid or a miniscus of fluid, reflections in the epi-fluorescence images can cause interference in data interpretation.

Example 9 Chemiluminescence Products

Two products available as chemiluminescence substrates for horse radish peroxidase can be compared as detection procedures for the MAPS assay. A MAPS plate is prepared as for Example 8, and incubated with biotinylated linker oligonucleotides. Then either alkaline phosphatase coupled to streptavidin (AlkPhos:SA) or HRP:SA is added, followed by washing and addition of either CDP-Star (Tropix) to the wells with AlkPhos:SA or ECL-Plus to the wells with HRP:SA. Labeling with SA derivatized enzymes and substrates is as suggested by the manufacturers for use in labeling of western blots. These two (as well as other available substrates) can both be used to assess oligonucleotide hybridization to MAPS plates.

Example 10 Resolution at 0.6 mm.

The resolution of the current system for MAPS assay is tested by preparing a MAPS plate with four different oligonucleotide anchors per well each spotted four times per well, with a pitch (center-to-center spacing) of 0.6 mm. Then either cy5-derivatized linkers or biotinylated linkers are hybridized and detected and scanned as above. For the epi-fluorescence measurement the resolution is higher (and pitch could likely be reduced). For the chemiluminescence detection procedure neighboring spots are not completely separated, yet at this spacing individual peaks may be resolved unambiguously by computer deconvolution.

Example 11 Test Nuclease Protection Protocol.

In an assay to test for the optimal conditions for hybridization and nuclease treatment for the nuclease protection protocol, the Nuclease Protection Assay kit from Ambion (Austin, Texas) is used to provide conditions, buffers and enzymes. Eight samples are prepared in one of three buffers. Hyb Buff 1 is 100% Hybridization Buffer (Ambion); Hyb

Buff 2 is 75% Hybridization Buffer and 25% Hybridization Dilution Buffer (Ambion); and Hyb Buff 3 is 50% of each. A 70-mer oligonucleotide that contains 60 residues complementary to a test mRNA is synthesized (Biosource International, Camarillo, CA) and labeled with Psoralen-fluorescein (Schleicher and Schuell, Keene, NH) following the protocol as suggested for labeling of Psoralen-biotin by Ambion. Briefly, protection fragment is diluted to 50 ug/ml in 20 µls of TE buffer(10 mM Tris, 1 mM EDTA, pH 8) boiled for 10 minutes, and rapidly cooled in ice water. Four µls of 130 ug/ml Psoralen-fluorescein in DMF is added, and the sample is illuminated for 45 minutes at 40° C with a hand-held long wavelength UV source. Free Psoralen-fluorescein is removed by extraction with saturated butanol. The mRNA used is GAPDH anti-sense mRNA, prepared from antisense plasmid (pTRI-GAPDH-Mouse antisense Control Template from Ambion) using T7 promoter and the MaxiScript kit (Ambion). The short protection fragment is the 60-mer complementary portion synthesized separately and similarly labeled. The sequences of the protection fragments are as follows:

Full length protection fragment: SEQ ID: 23
CGAGAAATATGACAACTCACTCAAGATTGTCAGCAATGCATCCTGCACCACCAACTGCTTGCT
TGTCTAA

Short protection fragment: SEQ ID: 24
CGAGAAATATGACAACTCACTCAAGATTGTCAGCAATGCATCCTGCACCACCAACTGCTT

Hybridizations are done by mixing protection fragments at 20 nM and GAPDH mRNA at 60 nM in 10 µls final volume for two hours at 22 ° C or 37 ° C . Following hybridization, 200 µls of a mixture of nucleases is added according to instructions from the manufacturer (Ambion Nuclease Protection Kit, 1:200 dilution of nuclease mixture) and incubated again at the same temperatures for 30 minutes. Hydrolysis is stopped with Hybridization Inhibition Buffer (Ambion), and oligonucleotides are pelleted and washed with Ethanol. 10 µls of 1x Gel Loading Buffer (Ambion) is added and oligonucleotides are separated on a 15% TBE-urea gel. The gel is swirled in running buffer for 30 minutes, put on a plastic plate and imaged with the Tundra using fluorescein filters for selecting excitation and emission wavelengths. The image is accumulated on the CCD array for 2 minutes. Best conditions

are those for samples incubated in Hyb Buff 2 at 37 ° C or in Hyb Buff 3 at 22 ° C. In these samples no detectable full-length protection fragment remains, and significant amounts of a portion of the full-length protection fragment at a size apparently the same as the short protection fragment are seen.

Example 12 mRNA Assay by NPA-MAPS. (see Figure 15)

The full NPA-MAPS protocol was used, with conditions for hybridization and nuclease treatment similar to those described in Example 11. Ten samples were run for the assay. All contained the same amount of the 70-mer oligonucleotide protection fragment and different amounts of GAPDH mRNA. Hybridization samples in 10 µls in 50% Hybridization Buffer and 50% Dilution Buffer containing 0.08 mg/ml Yeast RNA (Ambion) were heated to 90 ° C for 6 minutes, briefly centrifuged, heated to 70 ° C for 5 minutes, and allowed to cool to 19 ° C and incubated for 19 hours. 200 µls of nuclease mixture was then added to each sample for 30 minutes at 19° C. 60 µls was aliquoted from each sample for the MAPS assay. 2 µl of 10 N NaOH and 2 µl of 0.5 M EDTA was added, and the sample heated to 90° C for 15 minutes, 37 ° C for 15 minutes, and allowed to sit at room temperature for 20 minutes. Then samples were neutralized with 2 µl of 10 M HCl, and 12 µls of 20x SSC containing 2 M HEPES pH 7.5 and 200 nM biotinylated detector oligonucleotide specific for the protection fragment was added along with 1 µl of 10% SDS. Samples were mixed, heated to 80° C for 5 minutes, and two 35 µl aliquots of each sample were pipetted to two wells of a MAPS plate (each sample was split in two and run in duplicate on the MAPS plate). The plate had been prepared as for standard MAPS protocol, with self-assembled CY5-derivatized linker specific for the protection fragment already attached. The MAPS plate was covered and incubated at 50° C overnight, and detection and luminescence performed as described. In the last sample, no nucleases were added during the assay as a control to visualize how the protection fragment alone would be detected by MAPS. In the lower portion of the figure, the intensity scan (as analyzed by the imager) for the top row of wells is presented. The amount of GAPDH mRNA present in the sample (that is, the amount in each duplicate well after aliquoting to the MAPS plate) is listed in the figure.

The oligonucleotides used for the MAPS plates were as follows:

Anchor*: SEQ ID: 25
CGCCGGTCGAGCGTTGTGGGAGCGC

Linker** SEQ ID: 26
CTTGAGTGAGTTGTCATATTTCTCGGATACTGAGTGCGCTCCCACAACGCTCGACCGG
CG

Protection fragment (complementary to mouse antisense mRNA for GAPDH)
SEQ ID: 27
CGAGAAATATGACAACTCACTCAAGATTGTCAGCAATGCATCCTGCACCACCAACTG
CTTGCTTGTCTAA

Detector Oligonucloeotide*** – labeled at 5' end with biotin SEQ ID: 28
AAGCAGTTGGTGGTGCAGGATGCAT

*Anchors were synthesized with C12 spacer with amide at the 5' end
**Linkers were synthesized with Cy5 attached at the 5' end
***Detector Oligonucleotides were synthesized with biotin attached at the 5' end

Example 13 Dilution Curve, NPA-MAPS (see Figure 16)

The data discussed in Example 12 and shown in Figure 15 were quantified and plotted as a dilution curve. The average and standard deviations for all eight spots of the two duplicate wells are plotted for each concentration of mRNA. A binding curve is superimposed, of the form:

Fraction Bound = Max Bound * 1 / (1 + IC_{50}/L)

where Max Bound is the maximum bound at saturation, Fraction Bound is the amount bound at ligand concentration, L, and the IC_{50} is the concentration of ligand at which the Fraction Bound is half of Max Bound. The curve is shown as red dots on the figure, drawn with a best fit value of IC_{50} = 4.2 femtomoles as labeled in the figure.

Example 14 NPA-MAPS assay of GAPDH mRNA in a total mouse liver RNA extract

A total mouse RNA extract is assayed for GAPDH mRNA with an NPA-MAPS assay and a dilution curve is made. Total RNA from mouse liver is prepared using a Qiagen kit. RNA is precipitated in 70% EtOH with 0.5 M Mg-Acetate, and resuspended in 10 µls

of 5x SSC with 0.05% SDS with 1.8 nM protection fragment. The protection fragment added is an oligonucleotide 70 bases long, 60 bases of which are complementary to mouse GAPDH. Either a fragment complementary to mouse GAPDH mRNA is used ("protection fragment"), or the complement of the sequence is used as a negative control ("antisense fragment").

RNA samples with protection fragments are heated to 90° C for 5 minutes, and hybridizations are done by bringing samples to 70° C and allowing them to cool slowly to room temperature over night. S1 nuclease (Promega) at 1:10 dilution is added in 30 µls of 1x S1 Nuclease Buffer (Promega) for 30 minutes at 19° C, and stopped by 1.6 µls of 10 N NaOH and 2.7 µls of 0.5 M EDTA. Samples are heated to 90° C for 15 minutes and then 37° C for 15 minutes to denature and destroy RNA, neutralized with 1.6 µls of 10 M HCl, and incubated on MAPS plates overnight in 5x SSC with 0.05% SDS supplemented with 200 mM HEPES pH 7.5 to which 30 nM biotinylated detection oligonucleotide is added. Washing and visualization with SA-HRP is done as described. The amount of signal decreases in parallel with decreasing amounts of mouse RNA (samples include 500, 170, 50, 5, or 0.5 µg of total mouse RNA. Two control samples are included to which no S1 nuclease is added. Signal is seen only for the complementary protection fragment.

Oligonucleotides used:

For Antisense Control (same oligonucleotides as for example 12):

Anchor*:

CGCCGGTCGAGCGTTGTGGGAGCGC SEQ ID: 25

Linker**

CTTGAGTGAGTTGTCATATTTCTCGGATACTGAGTGCGCTCCCACAACGCTCGACCGG CG SEQ ID: 26

Protection fragment (complementary to mouse antisense mRNA for GAPDH)

CGAGAAATATGACAACTCACTCAAGATTGTCAGCAATGCATCCTGCACCACCAACTG CTTGCTTGTCTAA SEQ ID: 27

Detector Oligonucloeotide***

AAGCAGTTGGTGGTGCAGGATGCAT SEQ ID: 28

For Sense GAPDH mRNA samples:

Anchor*: SEQ ID: 25

CGCCGGTCGAGCGTTGTGGGAGCGC

Linker** SEQ ID: 29

ATGCATCCTGCACCACCAACTGCTTGATACTGAGTGCGCTCCCACAACGCTCGACCGGCG

Protection fragment (complementary to mouse mRNA for GAPDH): SEQ ID: 30

AAGCAGTTGGTGGTGCAGGATGCATTGCTGACAATCTTGAGTGAGTTGTCATATTTCT CGGCTTGTCTAA

Detector Oligonucleotide*** SEQ ID: 31

CGAGAAATATGACAACTCACTCAAG

*Anchors were synthesized with C12 spacer with amide at the 5' end

**Linkers were synthesized with Cy5 attached at the 5' end

***Probes were synthesized with biotin attached at the 5' end

Example 15 **A Nuclease Protection MAPS Assay with Controls.**

mRNA is extracted from mouse liver and nuclease protection is performed essentially as described in Example 14, except that the GADPH specific protection fragment comprises 60 nucleotides which are complementary to mouse GAPDH, followed by 15 "overhanging" nucleotides at the 3' end of the fragment which are not complementary to the target. After hybridization and nuclease digestion the remaining protection fragment is hybridized to a MAPS plate as indicated in Example 14, except that two different oligonucleotide detection fragments are used to detect the immobilized protection fragment. One detection fragment is complementary to the GAPDH-specific portion of the protection fragment, and the other, a control, is complementary to the 15 base overhang portion of the protection fragment. Each detection fragment is used on different replicate samples (i.e., in different wells), so that both detection fragments can be labeled with the same detection molecule. In the present example both fragments are labeled with HRP. Without the addition of nuclease, signals from both of the detection fragments are seen; whereas, when nuclease digestion is performed only the signal corresponding to the GAPDH sequences can be detected . The amount of GAPDH-specific signal is reduced relative to that observed in the absence of nuclease digestion, because the protection fragment is added at excess relative to the amount of GAPDH mRNA present. This allows the amount of GAPDH mRNA to be limiting to the protective hybridization, so that the amount of double-stranded hybrid formed (and therefore the amount of protection fragment that is protected from the nuclease) reflects the amount of mRNA. When no mRNA is included in the reaction

mixture, neither signal can be detected when nucleases are added. The above findings demonstrate that the hybridization and digestion steps of the assay occurred as desired.

When protection fragments corresponding to a variety of targets are included in a given assay, each of the protection fragments can comprise the same 15 base overhang portion. This allows for one detection fragment to be used to test for remaining overhang for all samples.

Example 16 **A transcription assay screening for compounds that may alter the expression of genes that are correlative with a disease state.**

A cell line derived from a human tumor is used. It is found to express 30 genes at higher levels than do normal cells. (That is, these 30 genes are being used more than in normal cells, to make mRNA and then to make the protein for which the genes are the instructions. A transcription assay measures how much the genes are being used by measuring how much mRNA for each gene is present.) Using a nuclease protection assay on MAPS plates (NPA-MAPS), 8800 chemical compounds are tested to see if growing the cells in the presence of the compounds can reduce the expression of some of the 30 correlative genes without affecting the expression of six normal (constitutive, "housekeeping") genes. Any compounds having that effect might be useful in the future development of drugs for treating this kind of tumor.

About 10,000 to 100,000 cells are added to each well of 100 96-well polystyrene plates and the cells are grown for 2 days until they cover the surface of each well. For 8 wells of each plate, the cells are left to grow without additions. To the remaining 88 wells of each plate, a different chemical compound is added so that the effect of it alone can be tested. For the 100 plates used at one time, 8800 compounds can be tested or screened. The cells are grown for 24 hours in the presence of the compounds, and then the cells are harvested for assay. The cells in each plate are treated according to the instructions for preparing RNA in samples from 96-well plates (for example according to the Qiagen RNeasy 96 kit). After the RNA is prepared, the amount of each of 36 different mRNA species is quantified by the NPA-MAPS approach, including the 30 correlative genes and

6 normal "housekeeping" genes. 36 DNA oligonucleotide protection fragments, each corresponding to one of the genes of interest, are added to each well and allowed to hybridize under selected stringent conditions to their target mRNA sequences. Then S1 nuclease is added to destroy excess unhybridized DNA, and the samples are treated chemically to destroy the RNA as well. Left is the oligonucleotide protection fragment for each of the 36 genes in proportion to how much mRNA had been present in the treated cells for each sample.

One hundred 96-well plates, each of which comprises an array of a plurality of 36 different anchor oligonucleotides in each well, are prepared by adding to each well 36 different linker oligonucleotides. The linkers self-assemble on the surface of each well, converting the generic plates to MAPS plates comprising specific probes for each of the 36 oligonucleotide protection fragments. Each linker has a portion specific for one of the 36 anchors and a portion specific for a segment of one of the 36 protection oligonucleotides. The oligonucleotide sample from each well of the 100 sample plates is added to a corresponding well of the 100 MAPS plates. After hybridization under selected stringent conditions, a detection oligonucleotide for each target with a chemiluminescent enzyme attached is added, so that each specific spot of each well lights up in proportion to how much mRNA had been present in the sample. Any wells that show reduced amounts of correlative genes with no effect on the 6 house keeping genes are interesting. The compounds added to the cells for those samples are possible starting points to develop anti-tumor agents.

Example 17 Induced and constitutive gene expression.

RNA was prepared essentially as described in Example 14, from the livers of mice either not infected ("Control") or one hour after infection ("Infected") by adenovirus. 60 µgs of liver RNA was used for each sample, and samples were prepared in duplicate. Each assay well contained three sets of duplicate loci, corresponding to the three genes described above. Each locus contained an anchor, bound to a linker comprising a probe which was complementary to a protection fragment corresponding to one of the three genes. A nuclease protection MAPS assay was performed essentially as described in Figure 12, and

the images were collected and scanned as described. Shown are the raw image data collected and the intensity scans for duplicate wells for each of the three mRNA targets. The numbers over the scan lines are the integrated intensity values and standard deviations for each condition (n = 4). The house-keeping gene, GAPDH, not expected to change, showed a modest increase of 1.3-fold in the infected sample that was not statistically significant. The transcription of MIP-2 and c-jun was increased 4 and 6-fold, respectively. These findings demonstrate that two genes, MIP-2 and c-jun, exhibit enhanced expression in response to adenovirus infection, compared to a control, constitutively expressed gene - GAPDH.

Example 18 **An enzyme assay screening for compounds that selectively inhibit tyrosine or serine kinases (see Figure 17).**

Kinases are enzymes that attach a phosphate to proteins. Many have been shown to stimulate normal and neoplastic cell growth. Hence, compounds that inhibit specific kinases (but not all kinases) can be used to test whether the kinases are involved in pathology and, if so, to serve as starting points for pharmaceutical development. For example, five tyrosine kinases that are involved in stimulating cell growth or in regulating the inflammatory response are src, lck, fyn, Zap70, and yes. Each kinase has substrates that are partially identified, as short peptides that contain a tyrosine. Some of the kinase specificities overlap so that different kinases may phosphorylate some peptides equally but others preferentially. For the five kinases, 36 peptide substrates are selected that show a spectrum of specific and overlapping specificities.

One hundred 96-well plates are used; each well comprises 36 generic oligonucleotide anchors. 36 linkers are prepared to convert the generic oligonucleotide array (with anchors only) to arrays comprising peptide substrates. The 36 peptide substrates are synthesized and each is attached covalently through an amide bond, for example, to an oligonucleotide containing a 5' amino group. The oligonucleotides contain sequences that hybridize specifically to the anchors. The peptide/oligo linkers are self assembled on the surface by adding them to all wells of the MAPS plates.

For screening, the five kinases at appropriate concentrations (so that the rates of

phosphorylation of the substrates are balanced as much as possible) are added to each well along with one of 8800 different compounds to be tested. The compounds are tested for their ability to directly inhibit the isolated enzymes. The amount of phosphorylation of each arrayed peptide is detected by adding labeled antibodies that bind only to peptides that are phosphorylated on tyrosine. Any wells that show a reduction in some of the phosphotyrosine spots but not all of the spots are interesting. Compounds that had been added to those wells can be tested further as possible selective inhibitors of some of the kinases tested.

The scheme of the assay is shown in the top panel of Figure 17. A chimeric linker molecule is prepared in which a 25 base pair oligonucleotide complementary to one of the anchors is crosslinked to a peptide substrate of a tyrosine phosphokinase enzyme. The chimeric oligo-peptide substrate self-assembles onto an array of oligonucleotide anchors, the kinase enzyme is used to phosphorylate the peptide portion of the chimera, and after the enzyme reaction is allowed to proceed, the amount of phosphorylation of the peptide is determined by anti-phoshotyrosine or anti-phosphoserine antibodies with an attached detection fluorophore or enzyme.

The results of the assay are shown in the lower panel. The homobifunctional crosslinker, DSS (Pierce), was used to attach the 5′ amino group of an oligonucleotide linker to the N terminus of a peptide synthesized with a phosphorylated tyrosine. The sequence of the peptide in single-letter code was: TSEPQpYQPGENL (SEQ ID: 32), where pY represents phosphotyrosine. The chimera was either used directly or first brought to pH 14 for 60 minutes in order to partially hydrolyze the phosphate group from the tyrosine. The phosphorylated or partially dephosphorylated chimeric molecules were self-assembled onto complementary anchor molecules within a MAPS plate at the concentrations shown for one hour. After washing and blocking the wells with 0.3% BSA in SSPTP antiphosphotyrosine antibody crosslinked to HRP (antibody 4G10 from Upstate Biotechnology, Lake Placid, NY) was added at a 1:3000 dilution in SSPTP for one hour, and the amount of antibody attached detected with chemiluminescence substrate, Super Signal Blaze. The image shown was accumulated on the CCD array for 1 minute. As expected a difference was seen in the

amount of phosphate attached to the oligo-peptide. This difference is the basis for an assay measuring how active a series of kinases is when treated with different possible inhibitors.

Example 19 A binding assay for the detection of selective inhibitors of the interaction between SH2 domains and phosphorylated peptides.

SH2 domains serve as docking subunits of some growth regulatory proteins. The domains bind to phosphotyrosine containing proteins or peptides with imperfect specificity. That is, some phosphotyrosine peptides bind specifically to one or to few SH2 proteins while others bind widely to many SH2 proteins.

For this assay, the linkers are phosphorylated peptides covalently attached to oligonucleotides. The peptide moieties are selected for their ability to bind to a group of selected SH2 proteins. The linkers convert generic MAPS plates to plates with ligands specific for the group of SH2 proteins. 100 96-well MAPS plates bearing the ligands are generated. The proteins are isolated and labeled with, for example, a cy5 fluorescent molecule.

In order to screen for inhibitors of the SH2 domain/ phosphopeptide interaction, the group of labeled SH2 proteins is added to each well of the 100 96-well MAPS plates, and in each well a different test compound is added. Hence the effect of each compound individually on the interaction of the SH2 proteins with their phosphopeptide ligands is tested. The assay is to measure the fluorescence of bound SH2 protein associated with each surface-bound peptide linker. For any well showing reduced fluorescence at some spots but not all spots, the compound added can be further tested as a putative selective inhibitor of SH2 docking.

Example 20 High Throughput Screening (see Figure 22)

Shown is a high throughput MAPS plate demonstrating the detection of signal from 96 wells in a single experiment. Hybridization to the same oligonucleotide was measured with 16 replicates in 80 wells. As shown, the reproducibility of the 1280 hybridization assays was very high. The left-most and right-most columns served as controls to

standardize the signal for different concentrations of the oligonucleotide.

In a similar fashion, 16 different oligonucleotides can be tested in each well, and the test repeated in the 80 different wells of the plate. Of course, an even greater number of different oligonucleotides or other probes, (*e.g.*, 100 nucleotide probes) can be assayed in each well, and many plates can be tested simultaneously (*e.g.*, 100 plates, such as 96-well microtiter plates). The large number of assays which can be performed on each sample (*e.g.*, in the latter case, about 100 different assays) and the large number of samples which can be assayed simultaneously (*e.g.*, in the latter case, about 96 x 100, or 9600 different samples) provides for very high throughput.

Example 21 Preparation of amplified target (see Figure 23)

A PCR primer (Primer One) is attached to a solid support (*e.g.*, a bead or a reaction vessel) via a chemical modification that has been introduced at the 5' terminus of the primer oligonucleotide. The primer comprises, 5' to 3', the chemical modification, a restriction enzyme site, and a sequence that is complementary to a target of interest (*e.g.*, a cDNA copy of an mRNA of interest). The target is amplified by PCR, using as PCR primers the attached Primer One plus a Primer Two, which comprises, 5' to 3', a sequence that is specific for a detector oligonucleotide and a sequence that is complementary to a different portion of the target than that of Primer One. Following PCR amplification, the amplified target DNA is washed to remove excess reaction material and is released from the solid support by cleavage with a restriction enzyme specific for the restriction site on Primer One. The amplified primer is thus released into the liquid phase. Thermal and/or chemical procedures can be used to deactivate the restriction enzyme and to denature the double stranded DNA product. The released, single stranded DNA target molecules can then be contacted with a surface comprising anchors and/or linkers, and the target can be detected using detector oligonucleotides complementary to the detector-specific sequences of Primer Two.

Example 22 Preparation of amplified target

A PCR primer (Primer One) is attached to a solid support (*e.g.*, a bead or a reaction

vessel) via a chemical modification that has been introduced into the 5' terminus of the primer oligonucleotide. The primer comprises, 5' to 3', the chemical modification, a peptide sequence which can be cleaved by a protease, and a sequence which is complementary to a target of interest (*e.g.*, a cDNA copy of an mRNA of interest). Instead of a peptide, any other element which can be cleaved specifically can also be used. Following PCR amplification as described, *e.g.*, in Example 21, the PCR product, still attached to the solid support, is denatured and (optionally) washed, leaving behind a single stranded molecule attached to the support. The washed, attached, molecule can then be cleaved and released (*e.g.*, by treatment with an appropriate protease), and contacted with a surface comprising anchors and/or linkers. Alternatively, the strand of the amplified target which is released following denaturation can be contacted with the surface comprising anchors and/or linkers. In either case, only one strand of the amplified target is contacted (*e.g.*, hybridized) with a linker, so competition for hybridization from the opposite strand of the amplified target is eliminated and background is reduced. Linkers can be designed to be specific for either, or both, of the amplified target strands.

Example 23 **Assay with Detection Linkers and Reporter Agents** **(See Figure 24)**

A sample comprising an mRNA of interest is subjected to a nuclease protection procedure, using as a protection fragment an oligonucleotide which comprises a target specific moiety and a control overhang moiety, which is not complementary to the mRNA. Following nuclease digestion, the control overhang moiety can be cleaved off, as desired, as is illustrated in the left portion of the figure; or the overhang can fail to be digested, as is illustrated in the right portion of the figure. The resulting nuclease protection fragments are hybridized to a detection linker, which comprises a target-specific moiety and a control overhang-specific moiety. In the assay shown in the left part of the figure, the control overhang moiety of the detection linker remains unhybridized; by contrast, in the assay shown in the right part of the figure, the control overhang moiety of the detection linker hybridizes to the residual control overhang sequence of the protection fragment. In a subsequent step of the assay, a reporter reagent, which comprises a moiety that can interact

with control overhang-specific moiety of the detection linker, is allowed to interact with the complexes. In the assay shown in the left part of the figure, the reporter reagent hybridizes to the control overhang-specific moiety of the detection linker, which remains available for hybridization, and the complex can be detected by virtue of the signaling entity on the reporter reagent. By contrast, in the assay shown in the right part of the figure, the reporter reagent is unable to bind to the complex because the complementary sequences are not available for hybridization, so no signal is associated with the complex.

In many of the assays of this invention, a reporter reagent can interact with any sequence present in a detection linker, not limited to a sequence specific for a control overhang.

Example 24 Multiple Fluors (See Figure 25)

A region comprising five loci, A - E, is shown in Figure 25. Each locus comprises a different group of substantially identical anchors, anchors A - E. To the anchors at locus A are hybridized four different types of linkers, each of which comprises a moiety specific for anchor A. However, each of the anchors comprises a different target-specific moiety: for targets 1, 2, 3 or 4. Therefore, after hybridization of targets to the anchor/linker complexes, targets 1, 2, 3, and 4 can all become localized at locus A. Similarly, four different types of linkers can hybridize to locus B. Each linker comprises a moiety specific for anchor B, but the target-specific moieties are specific for targets 5, 6, 7 or 8. In a similar fashion, targets 9-12 can become associated with locus C, targets 13-16 at locus D, and targets 17-20 at locus E. If each of these targets is labeled, either directly or indirectly, with a different, independently detectable fluor, such as, *e.g.*, an upconvertable phosphore, one can independently detect all 20 targets at the five indicated loci.

Example 25 An Assay in High Throughput Format

In this example, a transcription assay of the invention is used to detect and quantify changes in a gene expression pattern, in a format ready for high throughput screening. All steps in the assay are performed robotically. Routine washing steps are not explicitly

described. All reactions are carried out by conventional procedures, which are known in the art and/or described herein.

THP-1 human monocytes are grown in 96-well V-bottom microtiter plates, with 50,000 or 150,000 cells/well. The cells are either untreated or are differentiated with phorbol 12- myristate 13- acetate (PMA) for 48 hours, followed by activation with lipopolysaccharide (LPS) for four hours. Following treatment, the cells are lysed in guanidium isothyocyanate and frozen until needed. mRNA is obtained using streptavidin-paramagnetic particles to which is bound biotin-poly dT. Alternately, total RNA is obtained by extraction with tri-reagent (Sigma Chemical Co., St. Louis, MO). Samples comprising either mRNA or total RNA are subjected to a nuclease protection procedure, using as DNA protection fragments a mixture of thirteen 60-mer single strand oligonucleotides, each of which comprises, 5' to 3', a 25-mer specific for one of the thirteen targets of interest (GAPDH, IL-1, TNF-α, cathepsin G, cox-2, cyclin-2, vimentin, LD78-β, HMG-17, osteopontin, β-thromboglobin, angiotensin or actin); a 10-mer spacer; a 25-mer specific for a common oligonucleotide detector probe; and a 15-mer common control overhang sequence. mRNA is thereby converted into a stochiometric amount of "corresponding DNA protection fragment," which serves as target in the assay. Control experiments in which these corresponding DNA protection fragments are incubated with a probe specific for the control overhang sequence show that substantially only sequences specific for the mRNA targets of interest are present in the corresponding protection fragments, as expected if nuclease digestion has occurred as desired.

Surfaces are prepared according to the methods of the invention. In each well of a 96-well DNA Bind Plate is placed an array of sixteen different 25-mer oligonucleotide anchors. Fourteen different anchor species are used. One anchor species is used at three of the four corners of the array, and 13 different anchor species are used, one each at the remaining locations in the array. The anchors are then hybridized, in a defined orthogonal pattern, to 60-mer oligonucleotide linkers, each of which comprises, 5' to 3', a 25-mer corresponding to one of the thirteen targets of interest, a 10-mer spacer, and a 25-mer specific for one of the anchors. Thus, in each of the multiply repeated 16-spot arrays, each

of the thirteen target-specific linkers is localized at a defined position (locus) in the array. See Figure 18 for an illustration of such an orthogonal array. Linkers corresponding to GAPDH, a constitutively expressed housekeeping gene which serves as an internal normalization control, are represented at three loci within each array. Control experiments indicate that the linkers, as well as the protection fragments and detector oligonucleotides used in the experiment, exhibit the desired specificity.

Samples comprising the mixtures of corresponding protection fragments prepared as described above are hybridized to the anchor/linker arrays. Samples derived from either untreated or induced cultures are used. The presence and amount of hybridized protection fragments at each locus is then detected by hybridization to labeled detector oligonucleotides. In order to normalize the amount of signal at each locus, the detector oligonucleotides are diluted with appropriate amounts of blocked oligomers, as described herein. The amount of signal at each locus is processed and normalized to the control GAPDH signals. The data obtained are reproducible in eight replicate samples, as well as in samples prepared from three independent experiments, performed on different days. A summary of the relative abundance of the thirteen transcripts in one experiment is shown in the Table below.

Relative Intensity (10^5 Cells/Well)

Gene	Control		Induced		Ratio
	Average	CV(n=16)	Average	CV(n=16)	
GAPDH	10110	7%	9833	9%	0.97
IL-1	527	36%	8124	38%	15.40
TNF	229	35%	2249	36%	9.80
GAPDH	9591	11%	10031	17%	1.05
Cathepsin G	10394	31%	19648	46%	1.89
COX-2	415	39%	3557	25%	8.58
Cyclin-2	1728	23%	2960	25%	1.71
Vimentin	25641	25%	71074	20%	2.77
LD78	1298	39%	13437	20%	10.35
HMG-17	8286	19%	2405	20%	0.29
Osteopontin	5604	42%	19053	46%	3.40
Thromboglobulin	-53	—	31761	23%	> 100
GAPDH	10299	13%	10136	12%	0.98
Angiotensin	3575	28%	6561	31%	1.84
Actin	12741	27%	21802	23%	1.71
(blank)	108	—	234	—	

Example 26 Computer Algorithm for Quantification of Multiple Array Plate Data

A preferred algorithm finds the position of all spots for a MAPS plate and automatically calculates a best-fit estimate of the amplitude of the signal for each data point. Preferably, the algorithm is implemented by a computer program.

1 – Select a small part of the image data, a 40x40 box, containing the intensity value of each pixel (picture element) of the image that includes the first well to be examined.

2 – Define a function that calculates the intensity expected at each pixel position, using 16 unknowns. The unknowns are:

The amplitudes of each of 13 different microarray spots (that is, how bright are the real signals at each position of the DNA array). There are 13 of these for the 4x4 (=16) spots within each well because some of the 16 spots are duplicates of the same target.

The x offset and the y offset defining the exact position of the 4x4 array of spots within this particular well

The background intensity of the picture within the well.

The function for each pixel position calculates the distance between the pixel and each spot, and adds up the contribution that each spot makes to the intensity observed at that pixel, by multiplying the spot amplitude by the impulse response function for the given distance. For the images used the impulse response function is defined by the sum of a Gaussian and a Lorentzian of appropriate (constant) radii.

3 – Start the fitting for the current well by guessing the values of the parameters quickly. To do this, calculate the average image intensity for 16 regions of the picture where the spots are expected to be. Subtract an offset from these 16 averages, and scale the difference by a constant factor. The offset and scaling constant are defined empirically. Rearrange the results to match up the 16 spots with the 13 amplitudes. For the background and offets use any small numbers.

4 – Optimize the fitted values (for the 16 unknowns) by curve fitting. In particular use a non-

linear least squares algorithm with Marquadt procedure for linearizing the fitting function, fitting 16 unknowns to 40x40 = 1600 equations (although of course not all equations are linearly independent).

5 – Use the x,y offset as fitted for the current well to estimate with improved precision where the grid will be for the next well of the microplate. It is expected to be 9 millimeters offset relative to the next neighbor well (converted to distance in the number of pixels by the magnification factor of the imaging system). Since the distance between wells is small relative to the size of the plate, using local estimates of position is most accurate.

6 – With the improved estimate of position, define a smaller box of image for the next well, moving to a 30x30 box of pixels. This makes the fitting proceed more quickly.

Go back to step 2 and repeat for each well.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make changes and modifications of the invention to adapt it to various usage and conditions.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The preceding preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The entire disclosure of all applications, patents and publications, cited above and in the figures are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A combination useful for the detection of one or more target(s) in a sample, which comprises, before the addition of said sample,

a) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

b) at least eight different oligonucleotide anchors, each in association with

c) a bifunctional linker which has a first portion that is specific for the oligonucleotide anchor, and a second portion that comprises a probe which is specific for said target(s).

2. A method of detecting at least one target, comprising contacting a sample which may comprise said target(s) with the combination of claim 1, under conditions effective for said target to bind to said combination.

3. A method of detecting at least one target, comprising

a) contacting a sample which may comprise said target(s) with the combination of claim 1, under conditions effective for said target to bind to said combination,

b) contacting said combination and any bound targets with a labeled detection probe, and

c) detecting said detection probe.

4. The method of claim 2, wherein said target is a protection fragment.

5. The method of claim 2, wherein said target(s) are amplified by PCR before said sample is contacted with said combination.

6. The method of claim 5, wherein said target(s) are amplified by PCR using

two PCR primers, either or both of which comprises a chemical modification which allows the primer to attach to a solid surface.

7. The method of claim 5, wherein said target(s) are amplified by PCR using two PCR primers, either or both of which comprises one or more restriction enzyme sites.

8. The method of claim 5, wherein said target(s) are amplified by PCR using two PCR primers, either or both of which comprises one or more peptide sequences which can be cleaved by a protease.

9. The method of claim 5, wherein said target(s) are amplified by PCR using two PCR primers, either or both of which comprises a sequence which is specific for said detection probe.

10. The method of claim 3, wherein said labeled detection probe comprises an upconverting phosphore.

11. The method of claim 3, wherein said combination and any bound targets are contacted with at least two different labeled detection probes, each of which labeled probe comprises a different upconverting phosphore.

12. The method of claim 4, further comprising

a) contacting said combination and any bound targets with a labeled detection probe, and

b) detecting said labeled detection probe,

wherein said labeled detection probe comprises an upconverting phosphore.

13. The method of claim 12, wherein said combination and any bound targets are contacted with at least two labeled detection probes, each of which labeled probe comprises

a different upconverting phosphore.

14. The method of claim 2, further comprising

a) contacting said combination and any bound targets with a detection linker, which comprises a moiety specific for said target and a moiety specific for a reporter reagent, which interacts with said detection linker and which comprises a signaling entity,

b) contacting said detection linker with said reporter reagent, and

c) detecting said signaling entity.

15. The method of claim 14, wherein said target is a nuclease protection fragment.

16. A method for detecting at least two targets, comprising

a) contacting a sample which may comprise said targets with the combination of claim 1, under conditions effective for said targets to bind to said combination,

b) contacting said combination and any bound targets with at least two detection linkers, each of which comprises a moiety specific for one of said targets and a moiety specific for a common reporter reagent,

c) contacting said detection linkers with said common reporter reagent, which interacts with said detection linkers and which comprises a signaling entity, and

c) detecting said signaling entity.

17. The method of claim 16, wherein said targets are nuclease protection fragments.

18. The method of claim 16, wherein said signaling entity comprises an upconverting phosphore.

19. The method of claim 2, for detecting at least two targets, further comprising,

a) incubating an RNA extract with two or more protection fragments under conditions which are effective for hybridization of said protection fragments to RNAs of interest in said extract, wherein each of said protection fragments comprises a common 3' overhanging sequence which is not specific for said RNAs,

b) subjecting said incubated extract to treatment with one or more nucleases effective for digesting substantially all nucleic acid other than the portion of said protection fragments which have hybridized to the RNAs of interest and, optionally, the portions of said RNAs which have been hybridized,

c) removing substantially all nucleic acid material other than said protection fragments which have hybridized to said RNAs of interest, to provide a sample containing, as targets, the protection fragments,

d) contacting said combination and any bound protection fragments with at least two detection linkers, each of which comprises a moiety specific for one of said targets and a moiety specific for said common 3' overhanging sequence,

e) contacting said detection linkers with a reporter reagent which is specific for said common reporter reagent and which comprises a signaling entity, and

f) detecting said signaling entity.

20. The method of claim 19, wherein said signaling entity comprises an upconverting phosphore.

21. The method of claim 19, wherein one or more of said detection linkers is diluted with blocked detection linker.

AMENDED CLAIMS

[received by the International Bureau on 07 May 2001 (07.05.01);
original claims 1-21 replaced by new claims 1-49 (11 pages)]

1. A method of detecting at least one target, comprising

a) contacting a sample which may comprise said target(s) with a combination which comprises, before the addition of said sample,

i) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

ii) at least eight different oligonucleotide anchors, each in association with

iii) a bifunctional linker which has a first portion that is specific for the oligonucleotide anchor, and a second portion that comprises a probe which is specific for said target(s),

wherein the target(s) is a protection fragment(s) and is amplified by PCR before said sample(s) is contacted with said combination,

under conditions effective for said target(s) to bind to said combination,

b) contacting said combination and any bound targets with a labeled detection probe, and

c) detecting said detection probe.

2. The method of claim 1, wherein said target(s) are amplified by PCR using two PCR primers, either or both of which comprises a chemical modification which allows the primer to attach to a solid surface.

3. The method of claim 1, wherein said target(s) are amplified by PCR using two PCR primers, either or both of which comprises one or more restriction enzyme sites.

4. The method of claim 1, wherein said target(s) are amplified by PCR using two PCR primers, either or both of which comprises one or more peptide sequences which can be cleaved by a protease.

5. The method of claim 1, wherein said target(s) are amplified by PCR using two PCR primers, either or both of which comprises a sequence which is specific for said detection probe.

6. A method of detecting at least one target, comprising

a) contacting a sample which may comprise said target(s) with a combination which comprises, before the addition of said sample,

i) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

ii) at least eight different oligonucleotide anchors, each in association with

iii) a bifunctional linker which has a first portion that is specific for the oligonucleotide anchor, and a second portion that comprises a probe which is specific for said target(s),

under conditions effective for said target(s) to bind to said combination,

b) contacting said combination and any bound targets with a labeled detection probe, and

c) detecting said detection probe,

wherein said labeled detection probe comprises an upconverting phosphore.

7. The method of claim 6, wherein said combination and any bound targets are contacted with at least two different labeled detection probes, each of which labeled probe comprises a different upconverting phosphore.

8. The method of claim 6, wherein said target(s) is a nuclease protection fragment.

9. The method of claim 8, wherein said combination and any bound targets are contacted with at least two labeled detection probes, each of which labeled probe comprises a different upconverting phosphore.

10. A method of detecting at least one target, comprising

a) contacting a sample which may comprise said target(s) with a combination which comprises, before the addition of said sample,

i) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

ii) at least eight different oligonucleotide anchors, each in association with

iii) a bifunctional linker which has a first portion that is specific for the oligonucleotide anchor, and a second portion that comprises a probe which is specific for said target(s),

under conditions effective for said target(s) to bind to said combination,

b) contacting said combination and any bound targets with a detection linker, which comprises a moiety specific for said target and a moiety specific for a reporter reagent, which interacts with said detection linker and which comprises a signaling entity,

c) contacting said detection linker with said reporter reagent, and

d) detecting said signaling entity.

11. The method of claim 10, wherein said target(s) is a nuclease protection fragment.

12. The method of claim 10, which is a method for detecting at least two targets, further comprising

b) contacting said combination and any bound targets with at least two detection linkers, each of which comprises a moiety specific for one of said targets and a moiety specific for a common reporter reagent,

c) contacting said detection linkers with said common reporter reagent, which interacts with said detection linkers and which comprises a signaling entity, and

d) detecting said signaling entity.

13. The method of claim 12, wherein said targets are nuclease protection fragments.

14. The method of claim 12, wherein said signaling entity comprises an upconverting phosphore.

15. A method of detecting at least two RNAs, comprising

a) incubating a sample which may comprise said RNAs with two or more protection fragments under conditions which are effective for hybridization of said protection fragments to said RNAs, wherein each of said protection fragments comprises a common 3' overhanging sequence which is not specific for said RNAs,

b) subjecting said incubated sample to treatment with one or more nucleases effective for digesting nucleic acid other than the portion of said protection fragments which have hybridized to said RNAs and, optionally, the portions of said RNAs which have been hybridized,

c) removing nucleic acid material other than said protection fragments which have hybridized to said RNAs, to provide a sample containing the protection fragments,

d) contacting said sample containing the protection fragments with a combination which comprises, before the addition of said sample,

i) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

ii) at least eight different oligonucleotide anchors, each in association with

iii) a bifunctional linker which has a first portion that is specific for the oligonucleotide anchor, and a second portion that comprises a probe which is specific for at least one of said protection fragments,

under conditions effective for said protection fragments to bind to said combination,

e) contacting said combination and any bound protection fragments with at least two detection linkers, each of which comprises a moiety specific for one of said protection fragments and a moiety specific for said common 3' overhanging sequence,

f) contacting said detection linkers with a reporter reagent which is specific for said common reporter reagent and which comprises a signaling entity, and

g) detecting said signaling entity.

16. The method of claim 15, wherein said signaling entity comprises an upconverting phosphore.

17. The method of claim 15, wherein one or more of said detection linkers is diluted with blocked detection linker.

18. A method of detecting at least one nucleic acid target, comprising

a) contacting a sample which may comprise said target(s) with a nuclease protection fragment(s) specific for and which binds to said target(s), exposing the sample to a nuclease effective to digest remaining single strand nucleic acid, and then contacting the resultant sample with a combination which comprises, before the addition of said sample,

i) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

ii) at least two different anchors, each in association with

iii) a bifunctional linker which has a first portion that is specific for the anchor, and a second portion that comprises a probe which is specific for said nuclease protection fragment(s),

under conditions effective for said nuclease protection fragment(s) to bind to said combination,

b) contacting said combination and any bound nuclease protection fragment(s) with at least one detection linker, which comprises a first moiety specific for one of said bound nuclease protection fragment(s) and a second moiety specific for a reporter reagent, and

c) detecting said detection linker(s).

19. The method of claim 18, wherein said reporter reagent interacts with said detection linker(s) and comprises a signaling entity, further comprising

d) contacting said detection linker(s) with said reporter reagent, and

e) detecting said signaling entity.

20. The method of claim 18, wherein the anchors are oligonucleotide anchors.

21. A method of detecting at least two nucleic acid targets, comprising

a) contacting a sample which may comprise said targets with nuclease protection fragments specific for and which bind to said targets, exposing the sample to a nuclease effective to digest remaining single strand nucleic acid, and then contacting the resultant sample with a combination which comprises, before the addition of said sample,

i) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

ii) at least two different anchors, each in association with

iii) a bifunctional linker which has a first portion that is specific for the anchor, and a second portion that comprises a probe which is specific for one of said nuclease protection fragments,

under conditions effective for said nuclease protection fragments to bind to said combination,

b) contacting said combination and any bound nuclease protection fragments with at least two detection linkers, each of which comprises a first moiety specific for one of said nuclease protection fragments and a second moiety specific for a common reporter reagent, and

c) detecting said detection linkers.

22. The method of claim 21, wherein said reporter reagent interacts with said detection linker(s) and comprises a signaling entity, further comprising

d) contacting said detection linker(s) with said reporter reagent, and

e) detecting said signaling entity.

23. The method of claim 21, wherein the anchors are oligonucleotide anchors.

24. A method of detecting at least two nucleic acid targets of interest in a sample which may comprise said targets, comprising

a) incubating said sample with two or more protection fragments under conditions which are effective for hybridization of said protection fragments to said nucleic acids of interest in said sample, wherein each of said protection fragments comprises a common 3' overhanging sequence which is not specific for said nucleic acids of interest,

b) subjecting said incubated sample to treatment with one or more nucleases effective for digesting nucleic acid other than the portions of said protection fragments which have hybridized to the nucleic acids of interest and, optionally, the portions of said nucleic acids of interest which have been hybridized,

c) removing nucleic acid material other than said protection fragments which have hybridized to said nucleic acids of interest, to provide a sample containing the protection fragments, then

d) contacting said sample containing the protection fragments with a combination which comprises, before the addition of said sample,

i) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

ii) at least two different anchors, each in association with

iii) a bifunctional linker which has a first portion that is specific for the anchor, and a second portion that comprises a probe which is specific for one of said protection fragments,

under conditions effective for said protection fragments to bind to said combination, and

e) contacting said combination and any bound protection fragments with at least two detection linkers, each of which comprises a first moiety specific for one of said protection fragments and a second moiety specific for said common 3' overhanging sequence.

25. The method of claim 24, further comprising

f) contacting said detection linkers with a reporter reagent which is specific for said common 3' overhanging sequence and which comprises a signaling entity, and

g) detecting said signaling entity.

26. The method of claim 24, wherein the anchors are oligonucleotide anchors.

27. The method of claim 26, wherein one or more of the detection linkers is diluted with blocked detection linker.

28. The method of claim 23, wherein at least one of said anchors is in association with a plurality of bifunctional linkers, each of which has a first portion that is specific for the anchor, and a second portion which comprises a probe which is specific for a different nuclease protection fragment.

29. The method of claim 18, wherein said anchors have been dissociated from bifunctional linkers having a different target specificity.

30. The method of claim 18, wherein said combination comprises a large number of said regions, and wherein the method is high throughput.

31. A kit useful for the detection of at least one nucleic acid target in a sample, which comprises

a) at least one nuclease protection fragment specific for at least one of said targets, but not for any of the oligonucleotide anchors in said kit,

b) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising at least two different oligonucleotide anchors,

c) a container comprising at least one bifunctional linker molecule, which has a first portion specific for at least one of said oligonucleotide anchors and a second portion that comprises a probe which is specific for, and in said detection binds to, at least one of said nuclease protection fragments, and

d) at least one detection linker, which has a first moiety specific for one of said nuclease protection fragments and a second moiety specific for a reporter reagent.

32. A kit useful for the detection of at least one nucleic acid target in a sample, which comprises:

a) at least one nuclease protection fragment specific for at least one of said targets, but not for any of the other oligonucleotides in said kit,

b) at least one bifunctional linker which has a first portion that is specific for an oligonucleotide anchor, and a second portion which is specific for, and in said detection binds to, at least one of said nuclease protection fragments, and

c) at least one detection linker, which has a first moiety specific for one of said nuclease protection fragments and a second moiety specific for a reporter reagent.

33. The method of claim 18, wherein each region comprises at least eight different anchors.

34. The method of claim 21, wherein each region comprises at least eight different anchors.

35. The method of claim 24, wherein each region comprises at least eight different anchors.

36. A method of detecting at least one nucleic acid target, comprising

a) contacting a sample which may comprise said target(s) with a nuclease protection fragment(s) specific for and which binds to said target(s) and exposing the resultant product to a nuclease effective to digest single strand nucleic acid, and then contacting the resultant sample with a combination which comprises, before the addition of said sample,

i) a surface comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

ii) at least two different anchors, each in association with

iii) a bifunctional linker which has a first portion that is specific for the anchor, and a second portion that comprises a probe which is specific for portions of said nucleic acid target(s) which are protected by said nuclease protection fragments,

under conditions effective for said protected portions(s) to bind to said combination,

b) contacting said combination and any bound protected portion(s) with at least one detection linker, which comprises a first moiety specific for one of said bound protected portion (s) and a second moiety specific for a reporter reagent, and

c) detecting said detection linker.

37. The method of claim 36, wherein said reporter reagent interacts with said detection linker(s) and comprises a signaling entity, further comprising

d) contacting said detection linker(s) with said reporter reagent, and

e) detecting said signaling entity.

38. The method of claim 36, wherein each region comprises at least eight different anchors.

39. A method of detecting at least one target, comprising

a) contacting a sample which may comprise said target(s) with a combination which comprises, before the addition of said sample,

i) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

ii) at least two different loci of anchors, the anchors at each locus each in association with

iii) a bifunctional linker which has a first portion that is specific for the anchor, and a second portion that comprises a probe which is specific for said target(s),

under conditions effective for said target(s) to bind to said combination, and

wherein two or more of the anchors located at at least one locus of a region are in association with different bifunctional linkers, having different target specificities.

40. The method of claim 39, further comprising

b) contacting said combination and any bound targets with at least one detection linker, which comprises a first moiety specific for one of said bound target(s) and a second moiety specific for a reporter reagent.

41. The method of claim 39, further comprising

c) contacting said combination and any bound targets with at least one detection probe.

42. The method of claim 41, wherein

a first detection probe binds to a first target bound to the combination at a first locus,

a second detection probe binds to a second target bound to the combination at the same locus,

and the first and second detection probes are detected simultaneously or sequentially.

43. The method of claim 39, wherein said target(s) is a nuclease protection fragment(s) specific for a nucleic acid(s) of interest.

44. The method of claim 40, wherein said target(s) is a nuclease protection fragment(s) specific for a nucleic acid(s) of interest.

45. The method of claim 42, wherein said target(s) is a nuclease protection fragment(s) specific for a nucleic acid(s) of interest.

46. The method of claim 39, wherein each region comprises at least eight different anchors.

47. The kit of claim 31, wherein each region comprises at least eight different anchors.

48. The kit of claim 31, further comprising

e) one or more nucleases effective for digesting single strand nucleic acid and/or the RNA strand of a DNA/RNA duplex.

49. A kit useful for the detection of at least one nucleic acid target, comprising

a) at least one nuclease protection fragment specific for said target(s), but not for any of the oligonucleotide anchors in said kit,

b) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising at least two different oligonucleotide anchors,

c) a container comprising at least one bifunctional linker molecule, which has a first portion specific for at least one of said oligonucleotide anchors and a second portion that comprises a probe which is specific for, and in said detection binds to, at least one of said nuclease protection fragments,

d) at least one detection linker, which has a first moiety specific for one of said nuclease protection fragments and a second moiety specific for a reporter reagent, and

e) one or more nucleases effective for digesting single strand nucleic acid and/or the RNA strand of a DNA/RNA duplex.

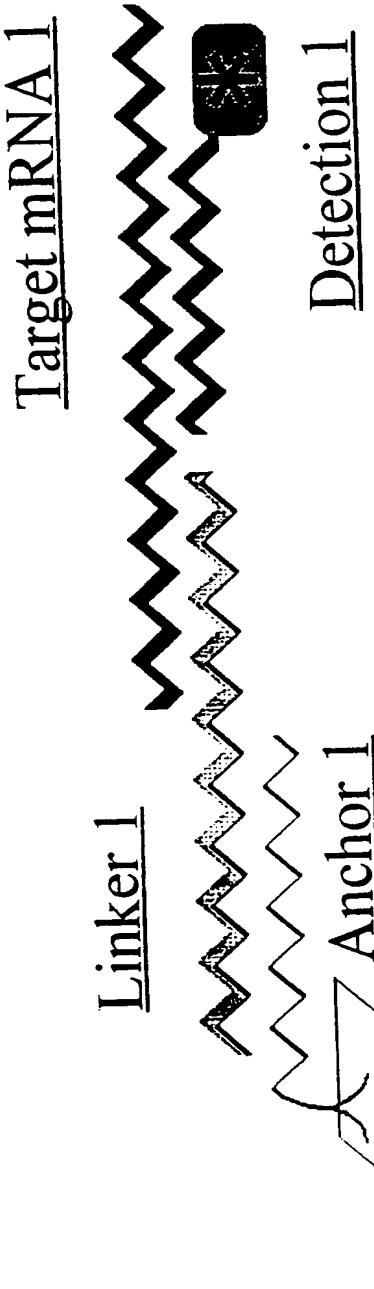


Fig 1

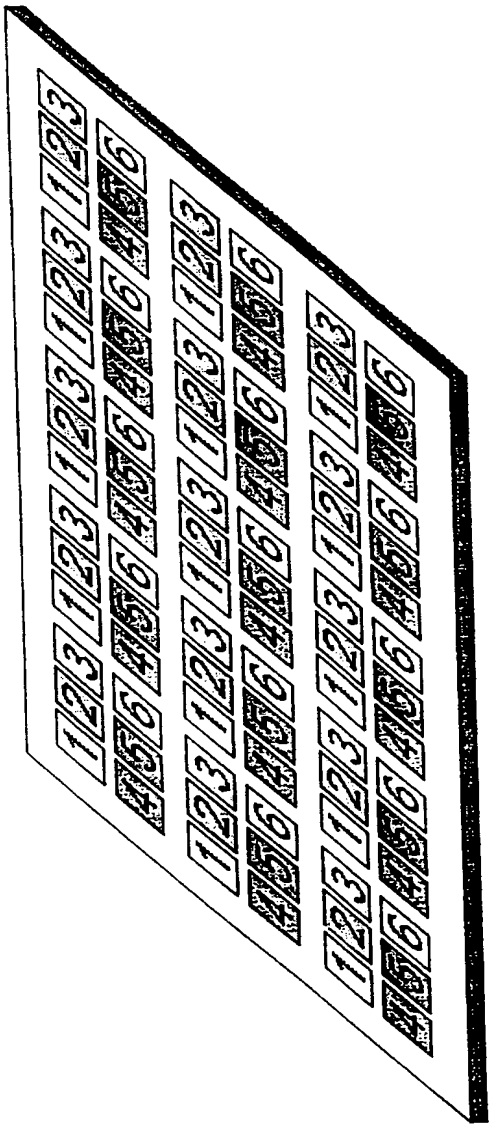


Fig 2

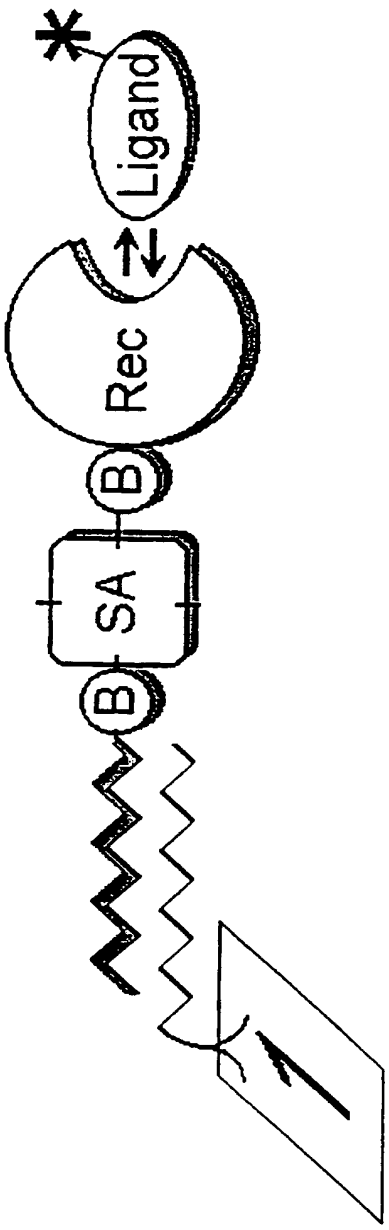


Fig 3

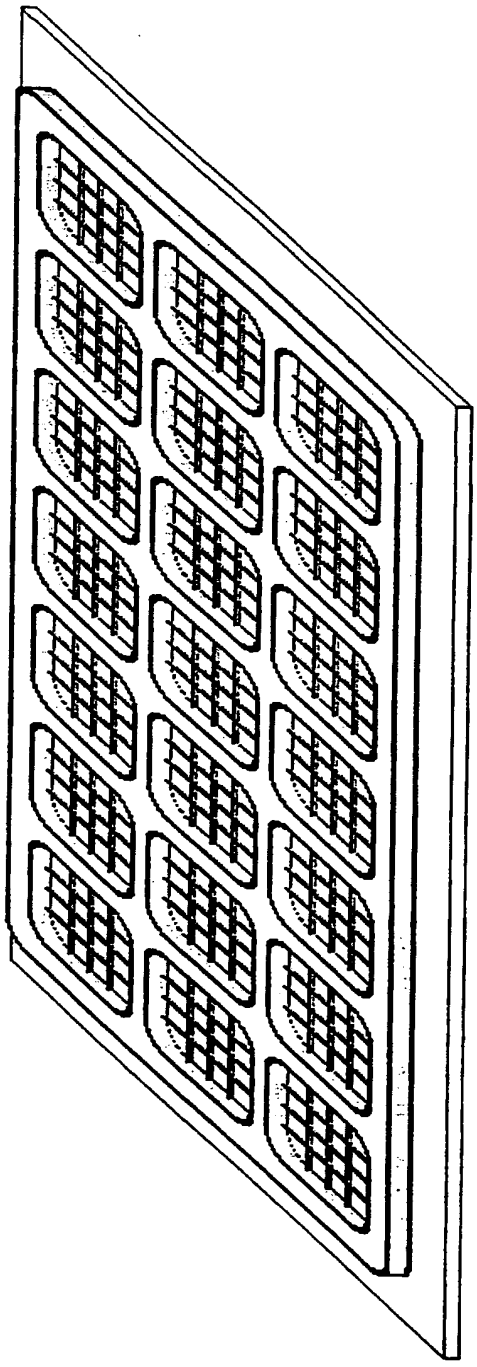


Fig 4

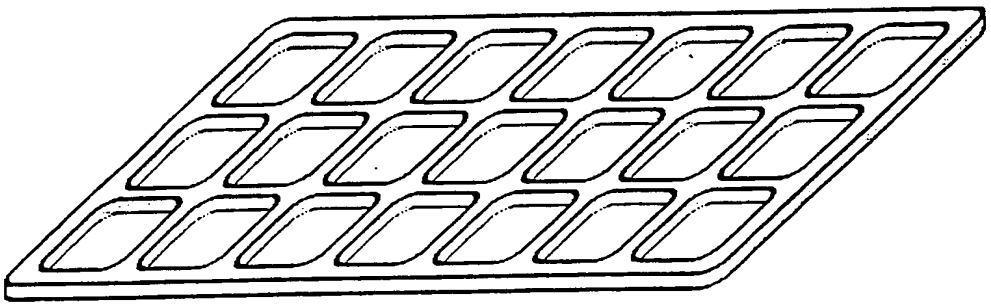


Fig 5a

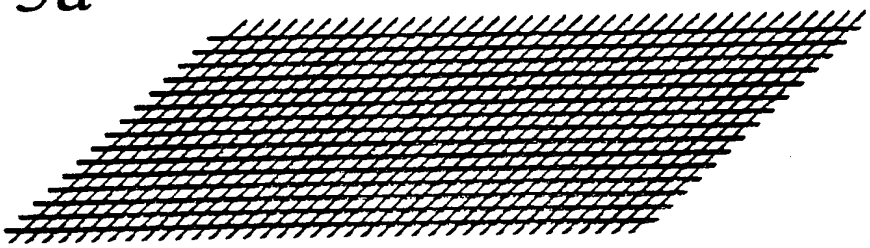


Fig 5b

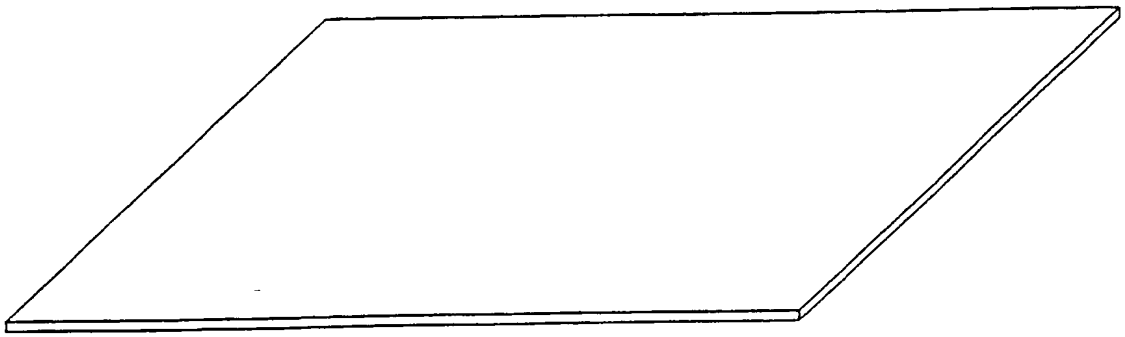


Fig 5c

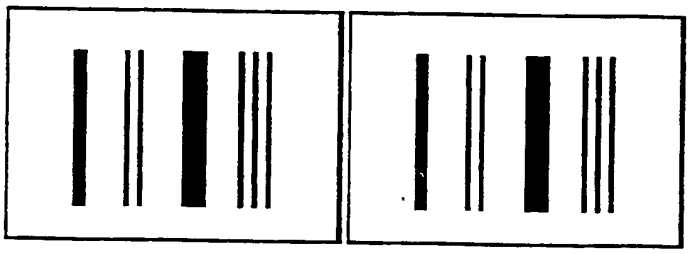


Fig 6

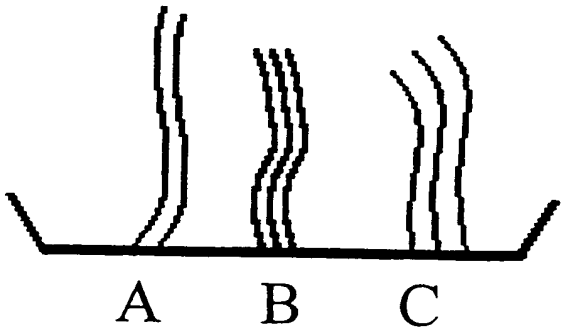


Fig 7

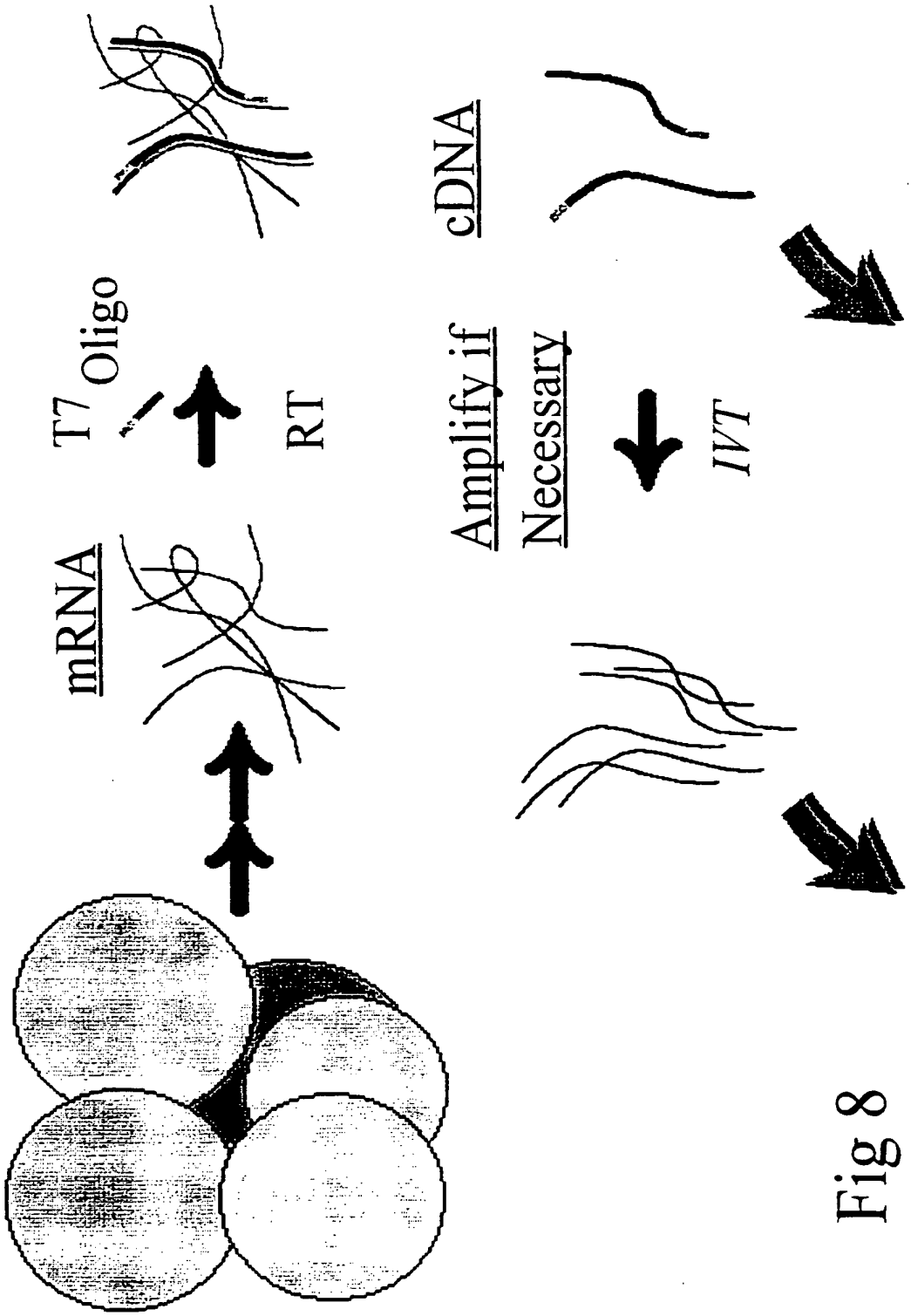


Fig 8

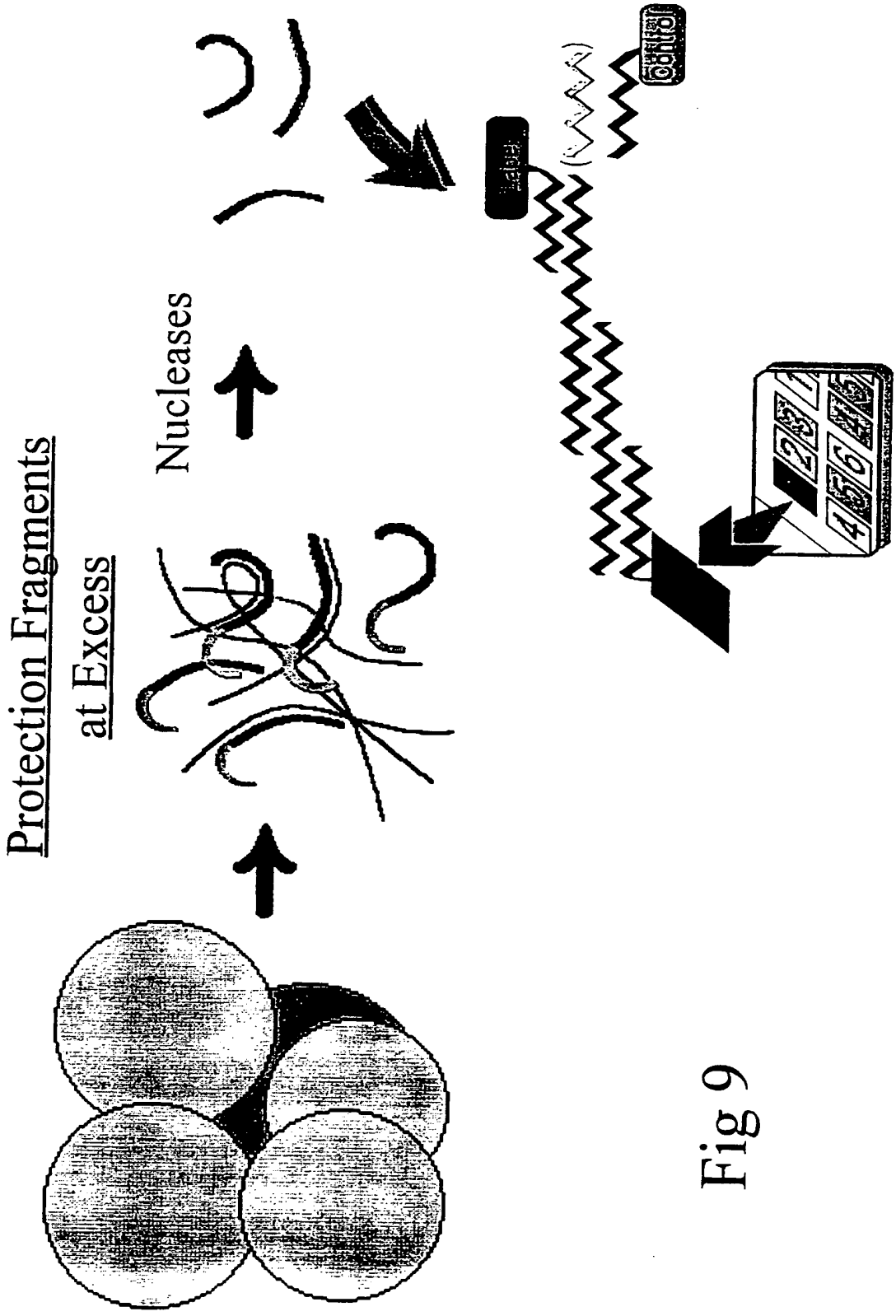


Fig 9

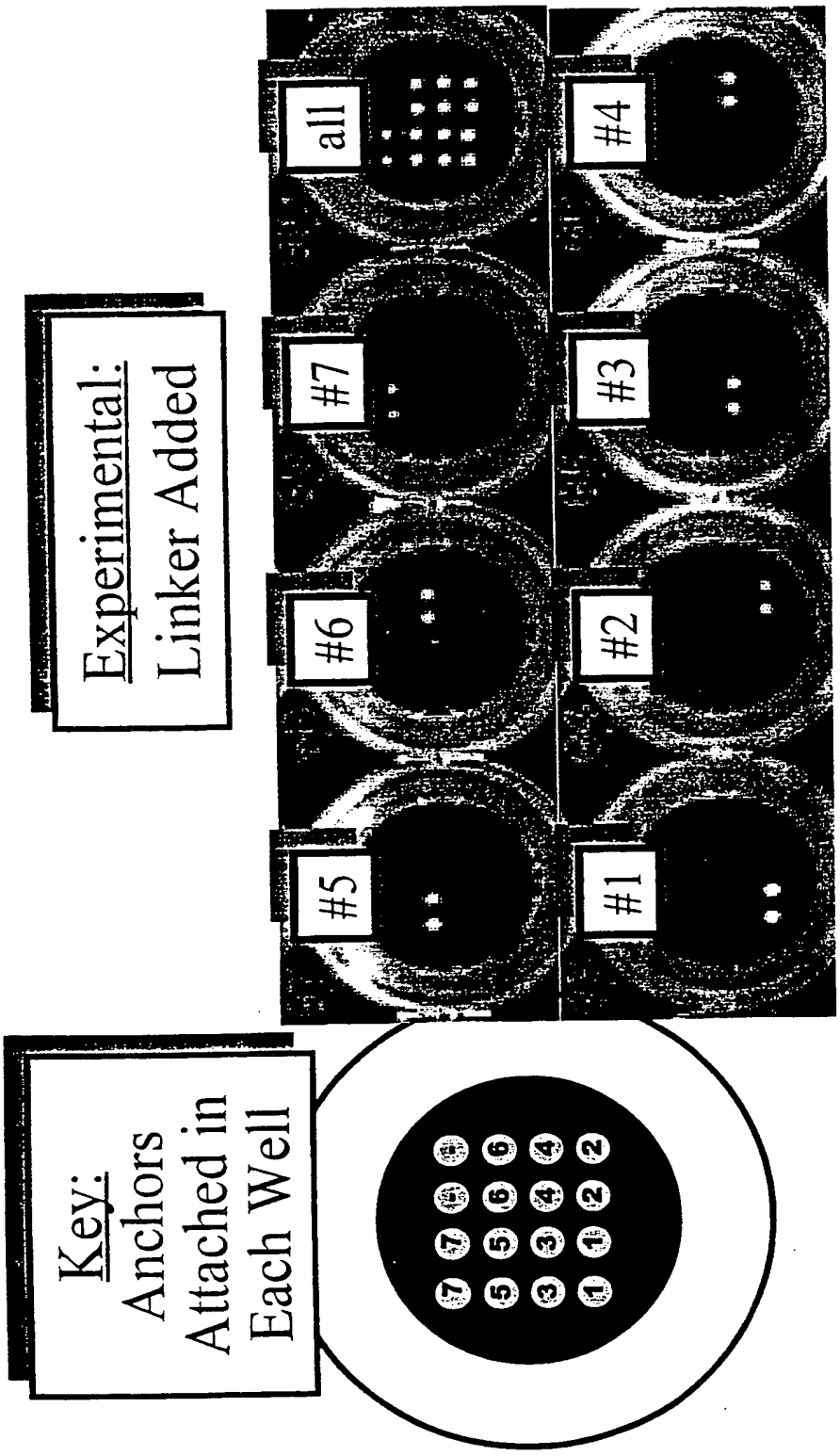


Fig 10

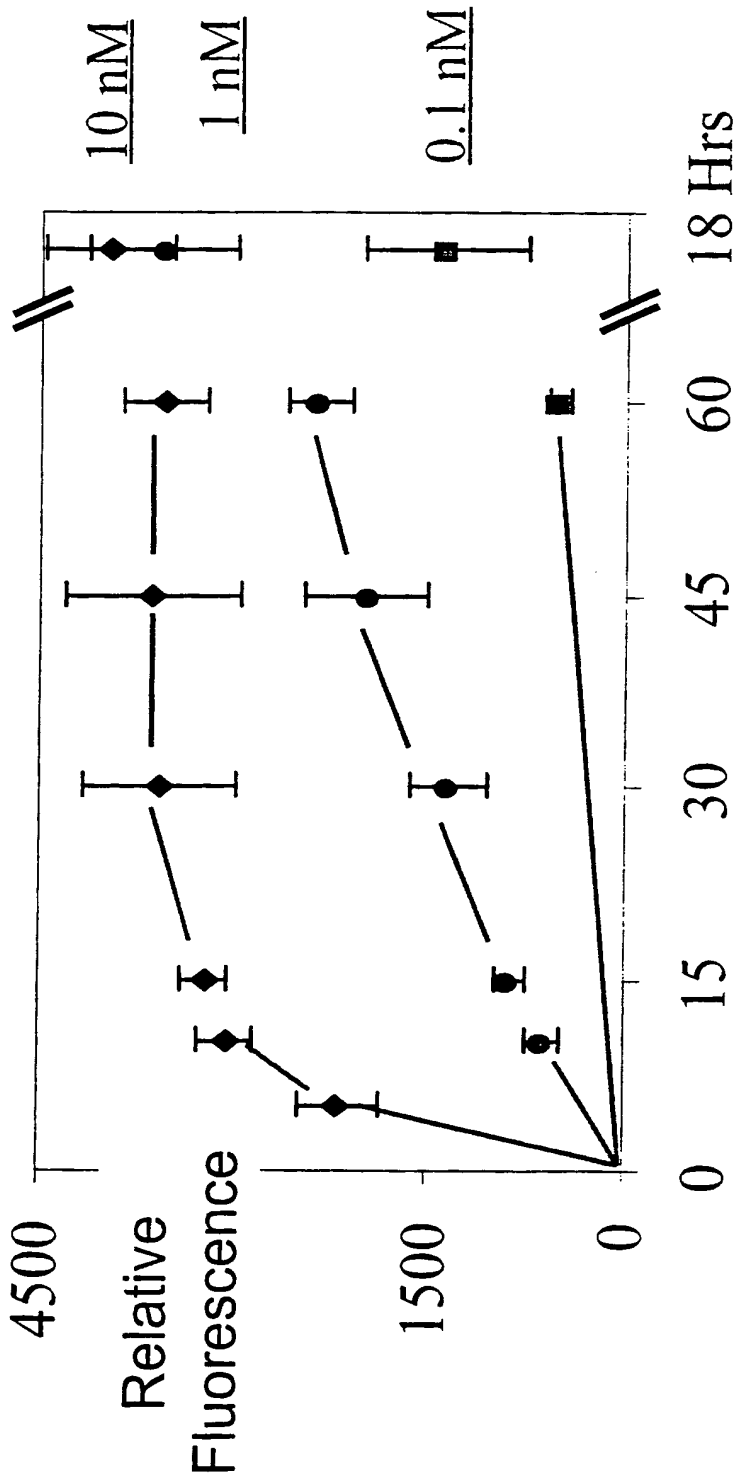


Fig 11

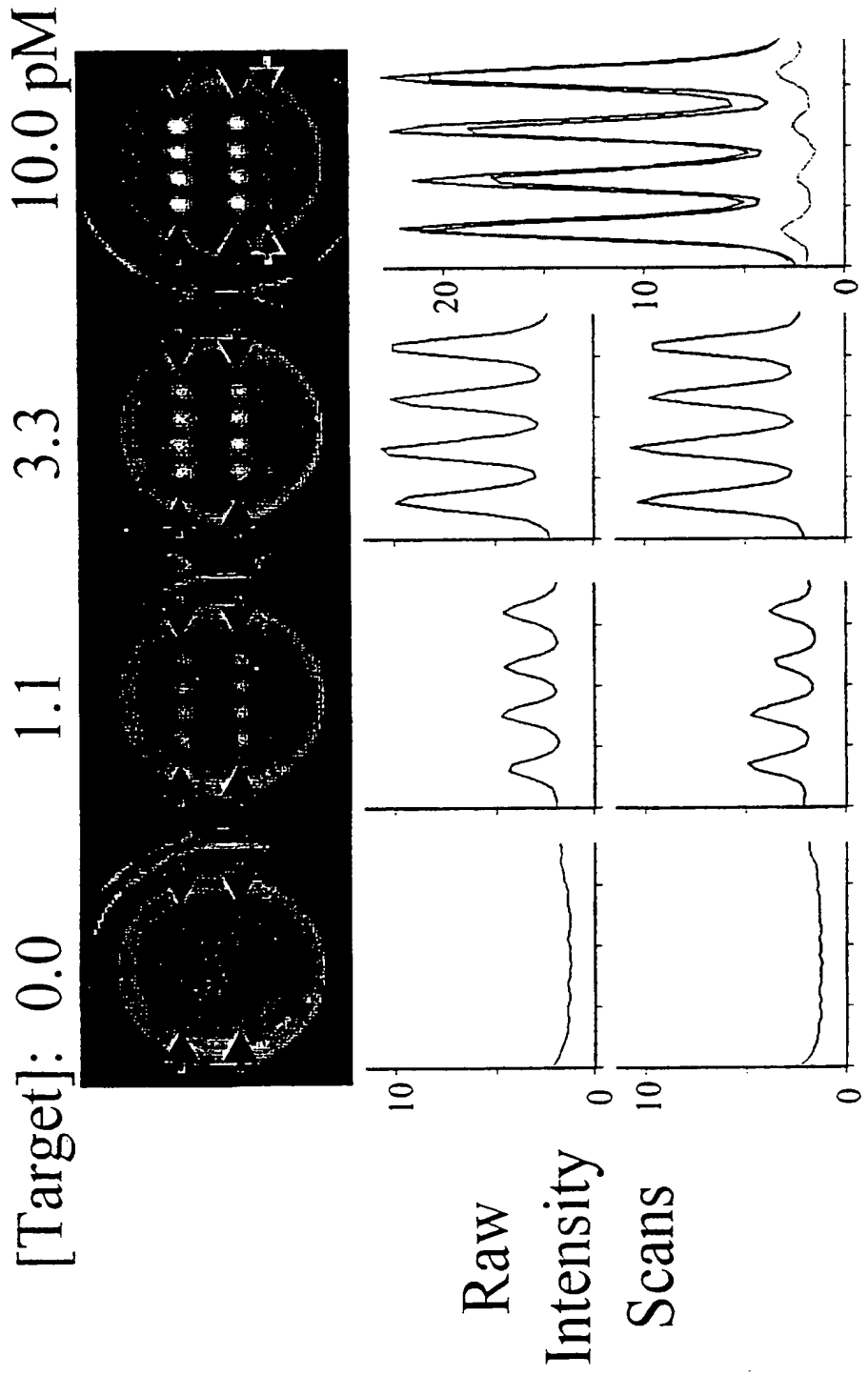


Fig 12

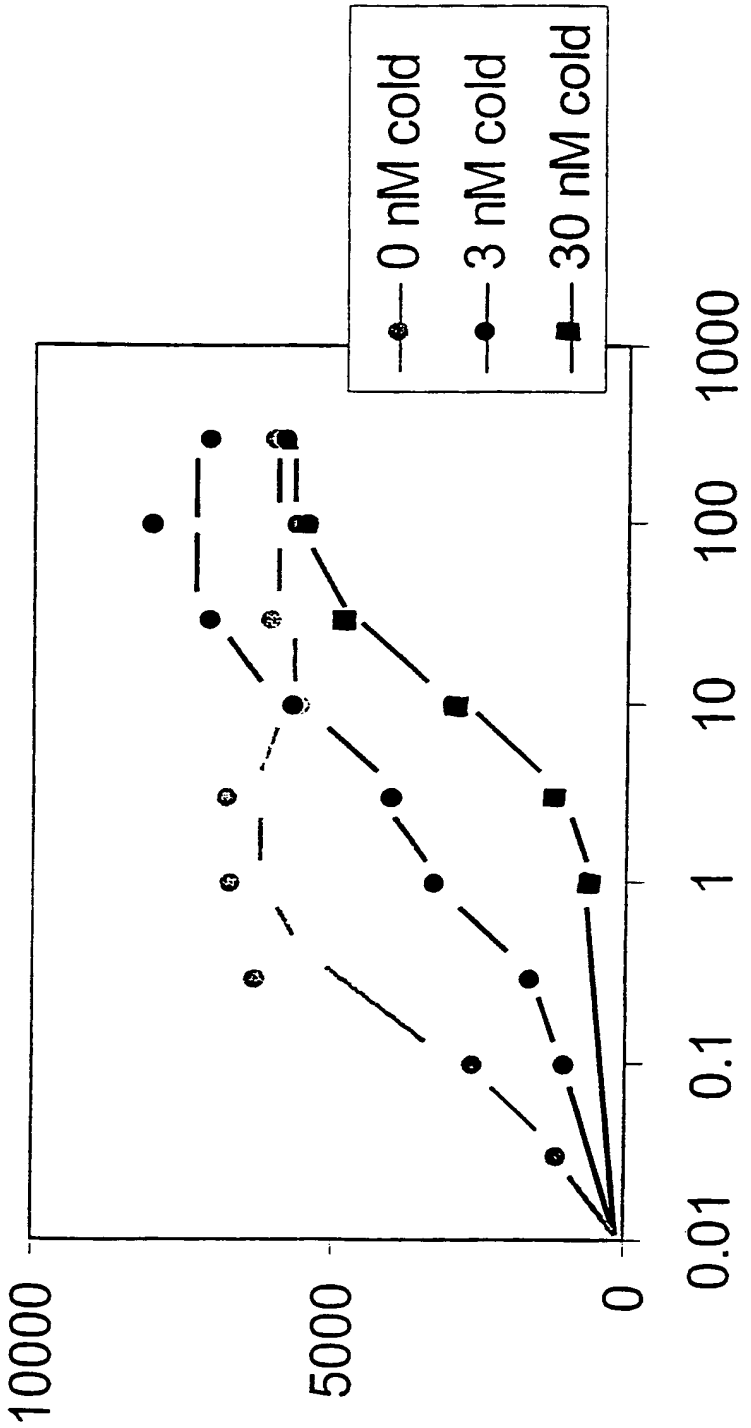


Fig 13

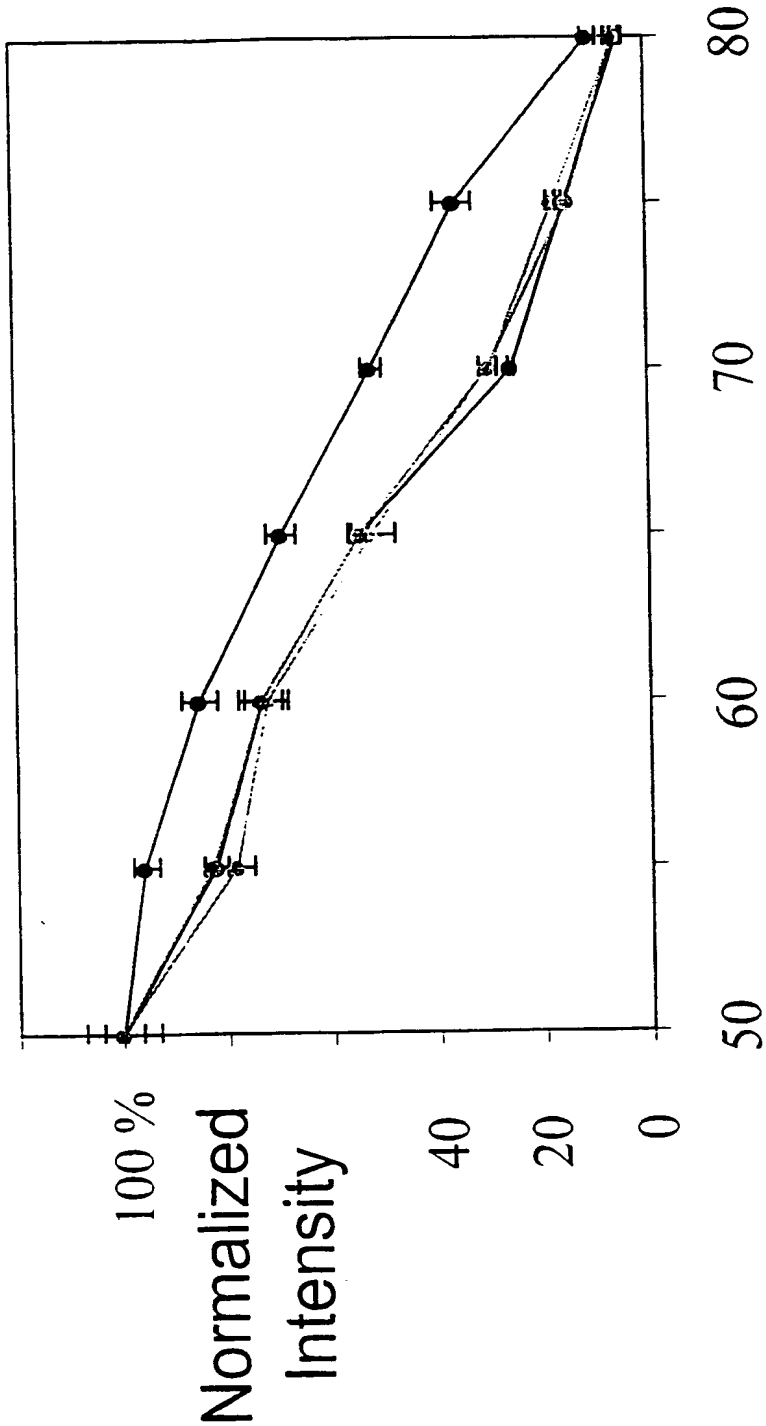


Fig 14

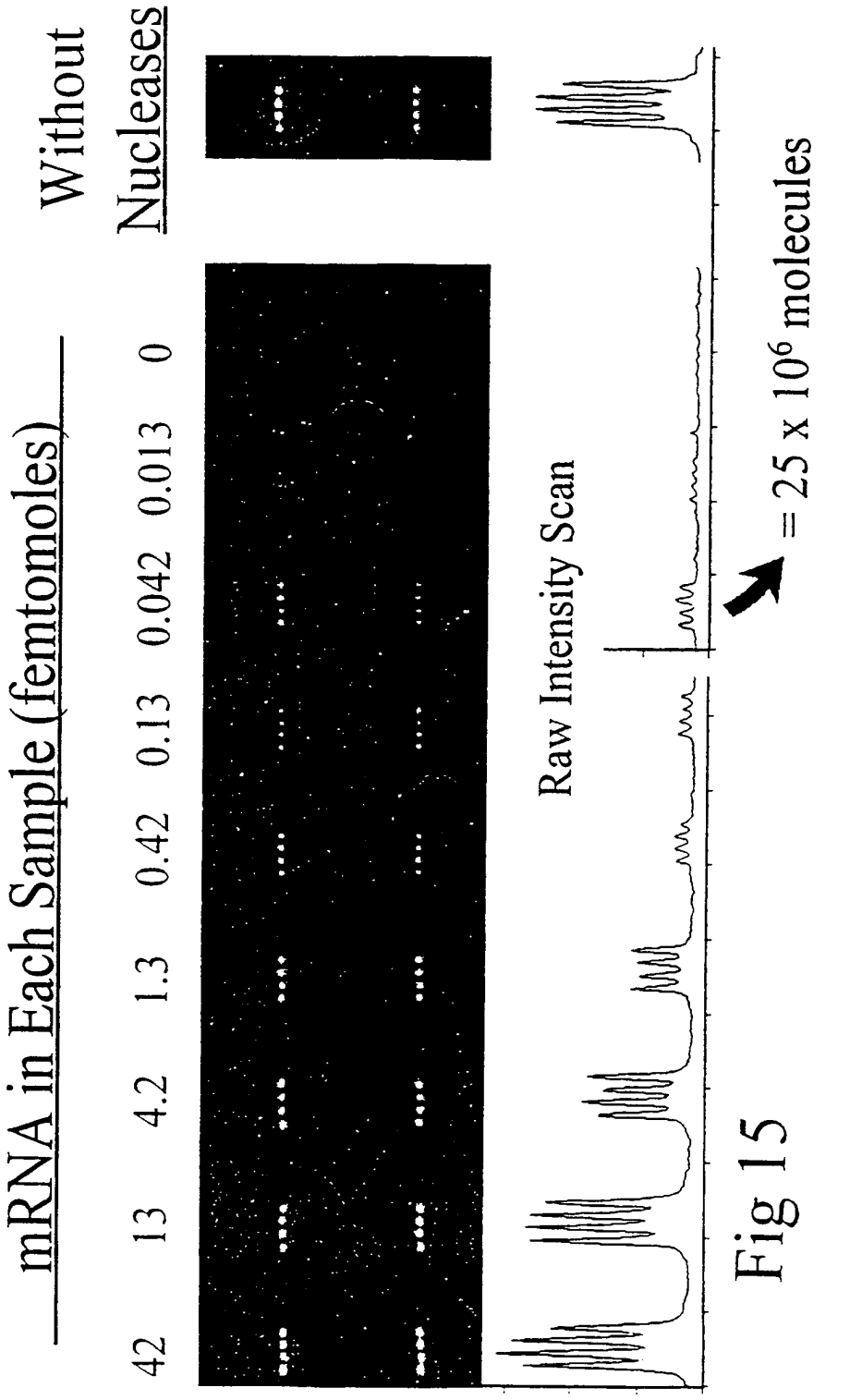


Fig 15

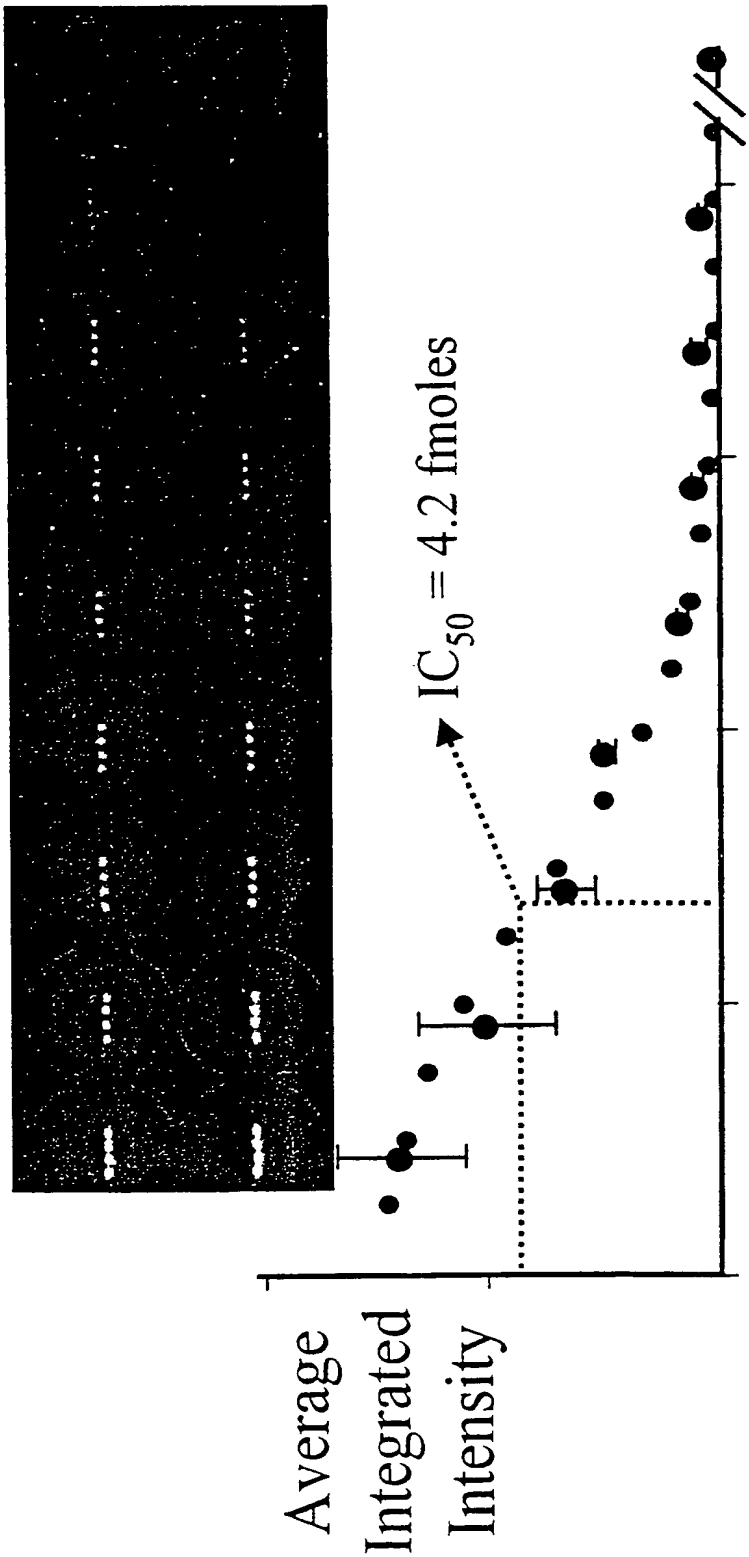
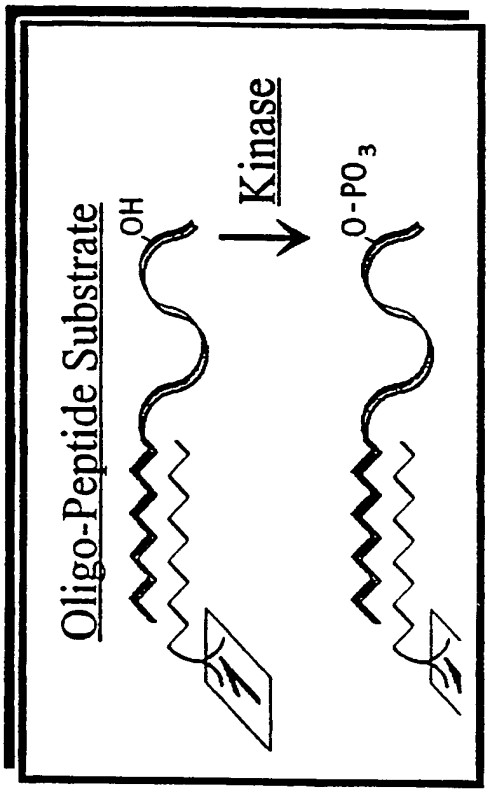


Fig 16



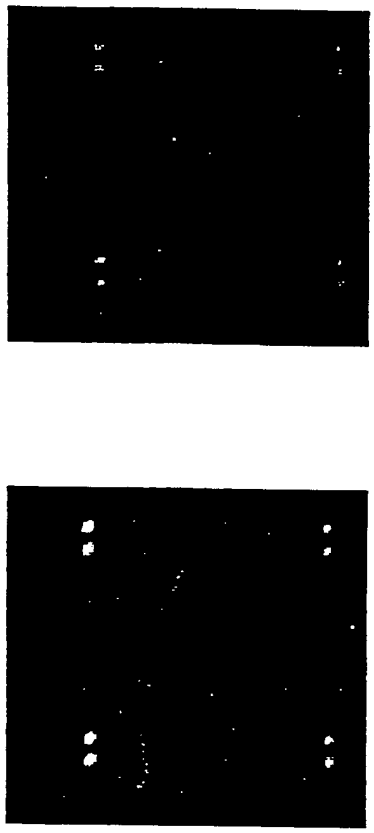


Fig 17

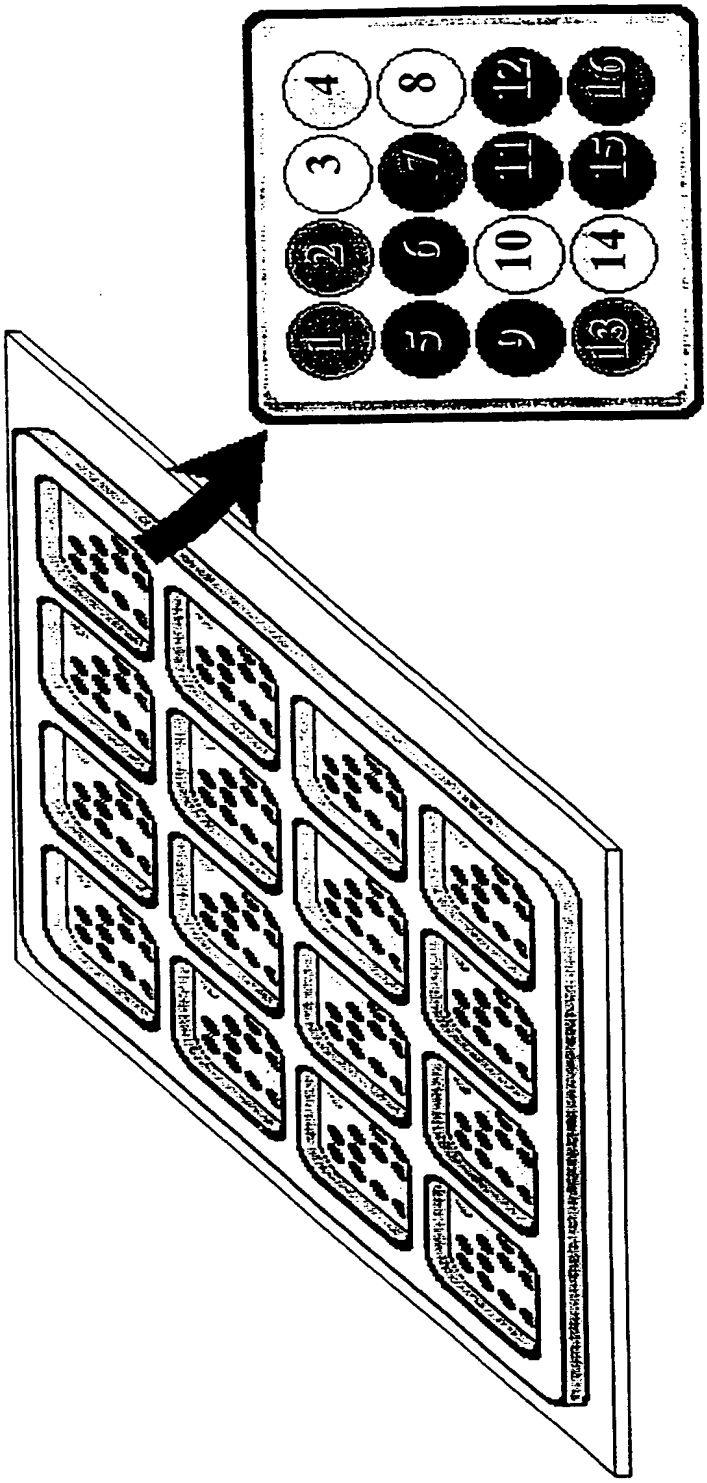


Fig 18

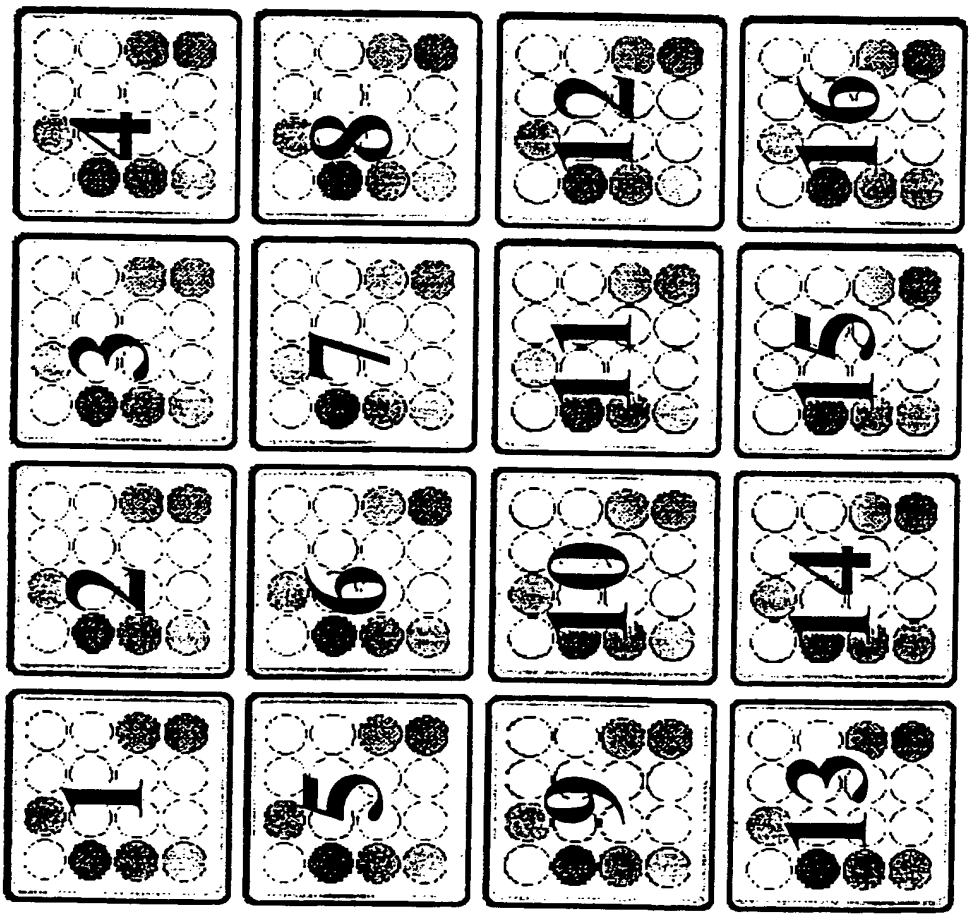


Fig 19

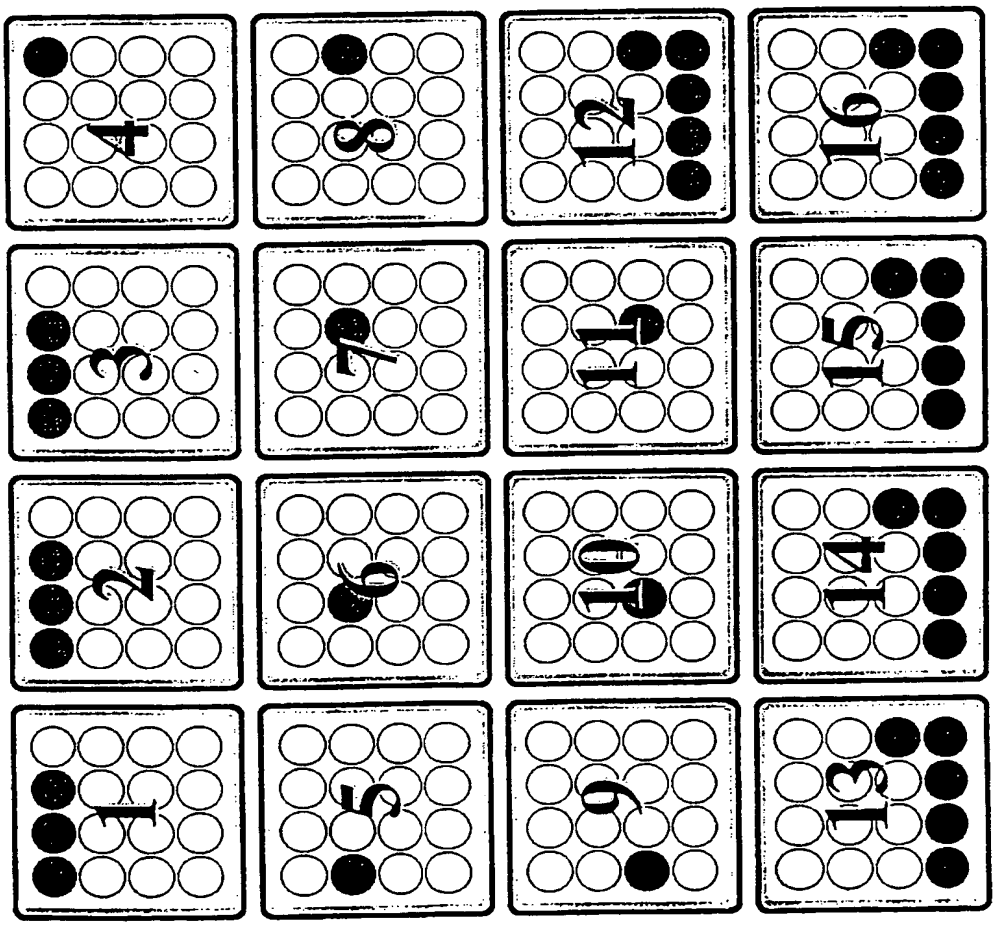


Fig 20a

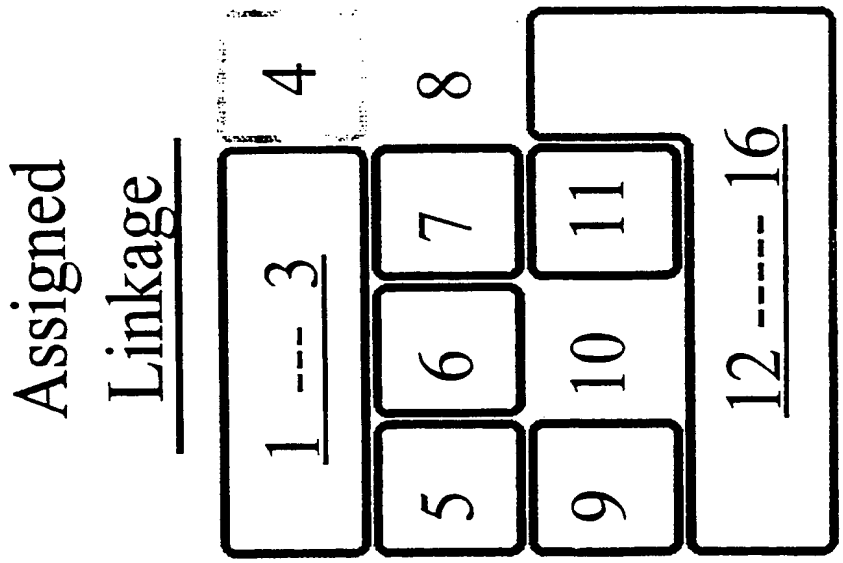


Fig 20b

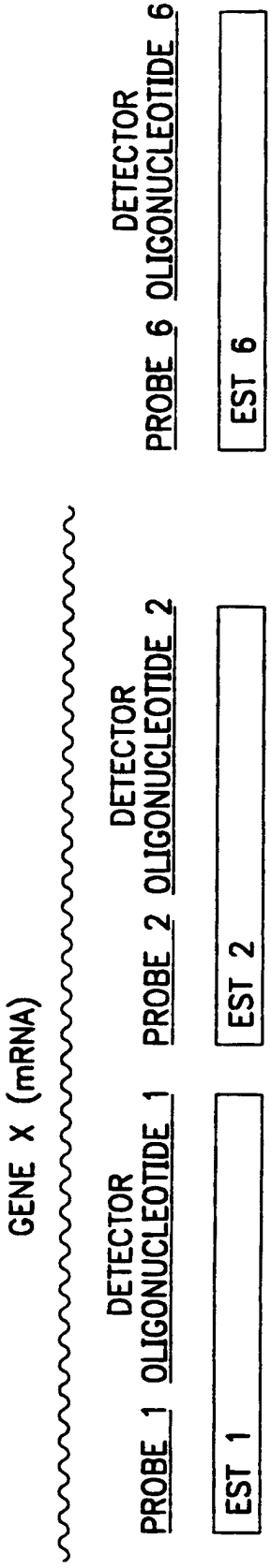


FIG. 21

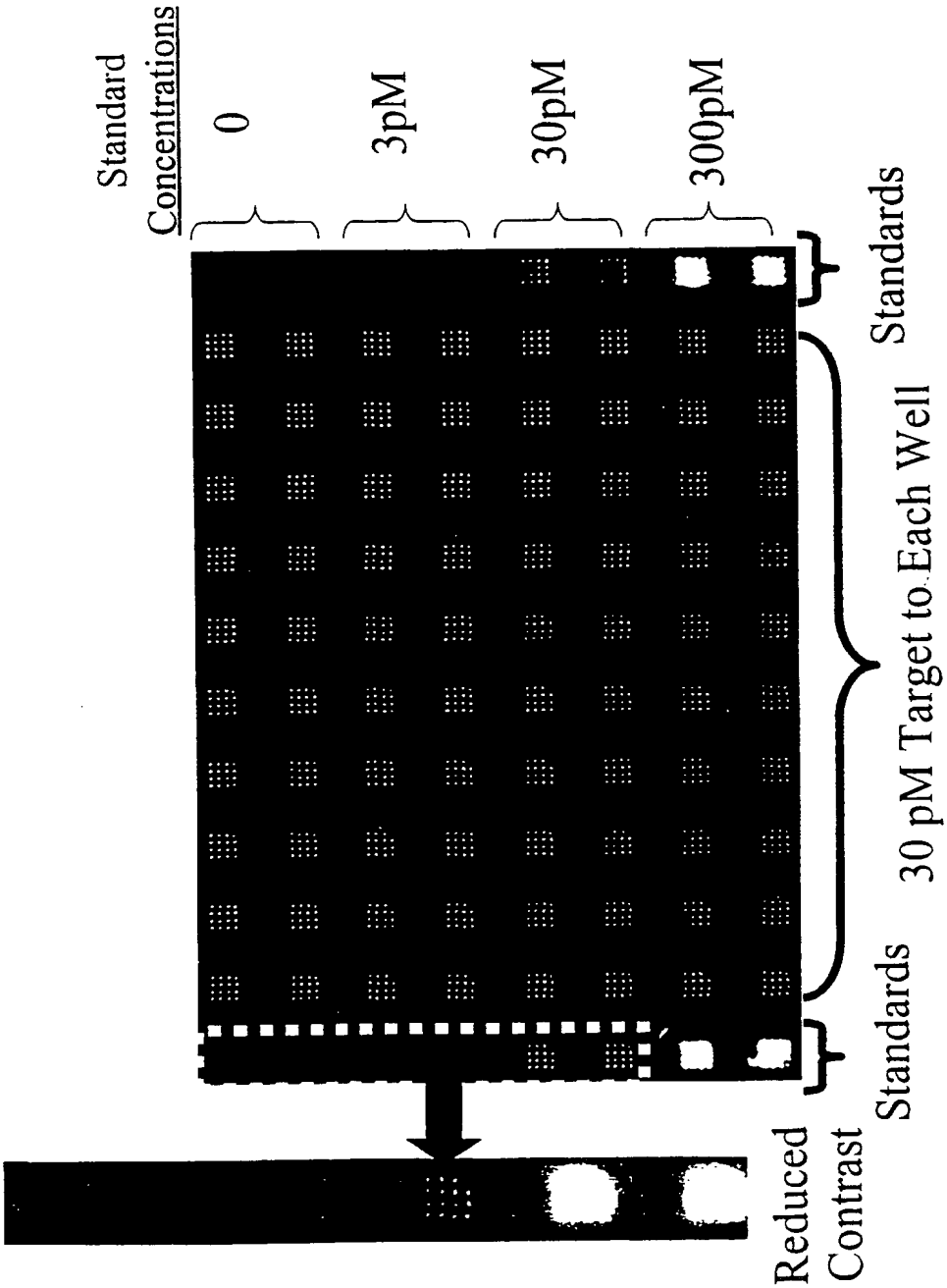


Fig 22

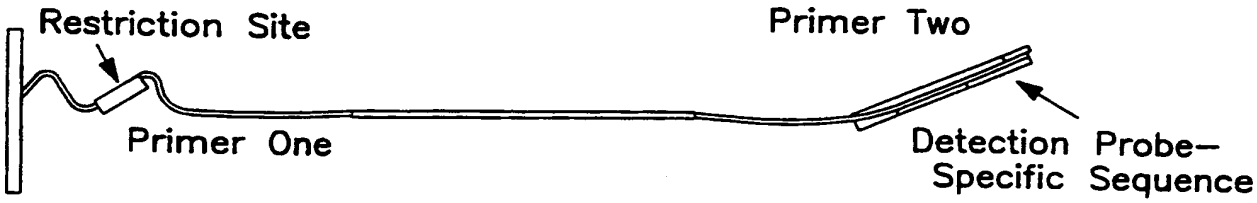


FIG. 23

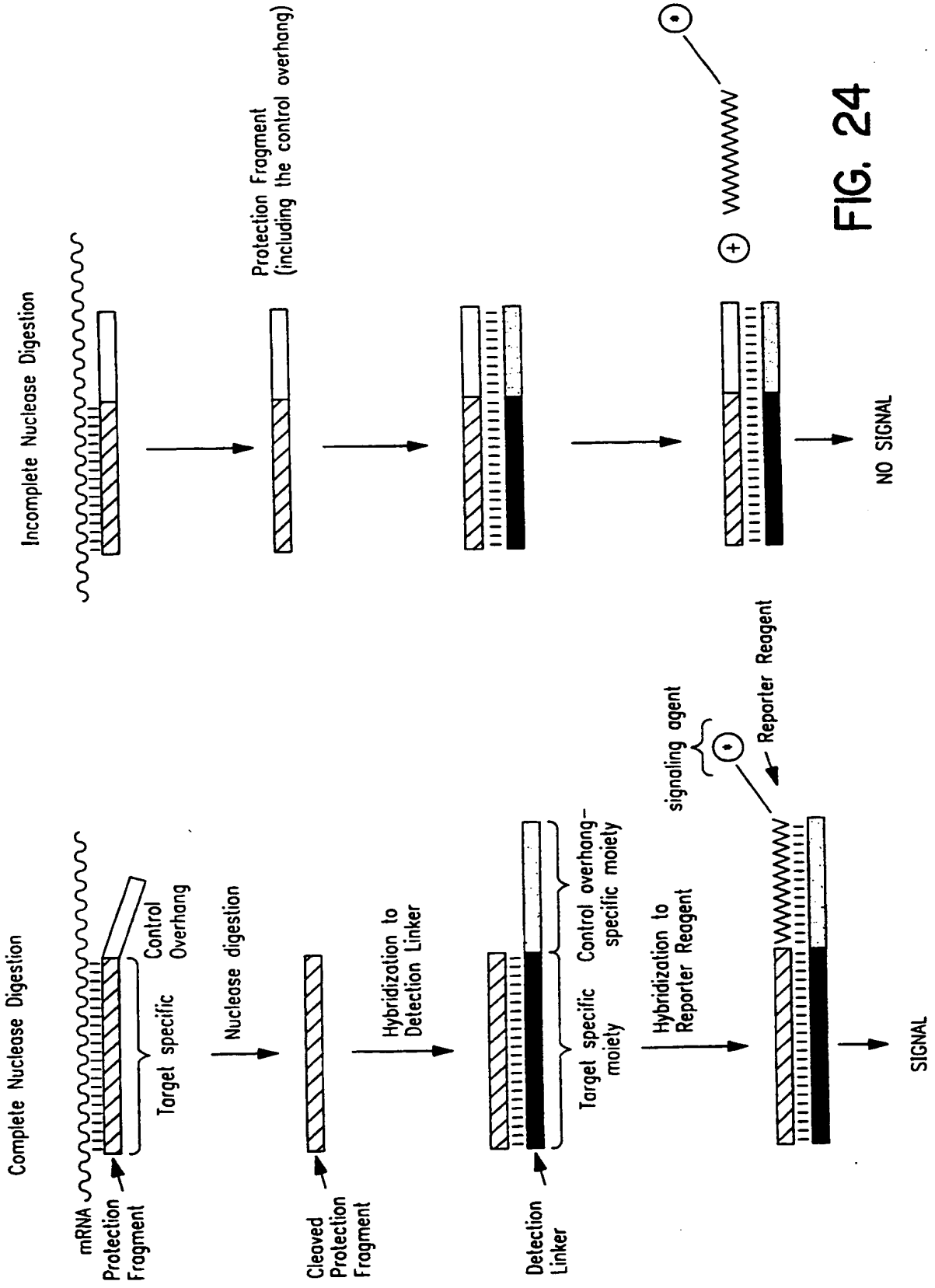


FIG. 24

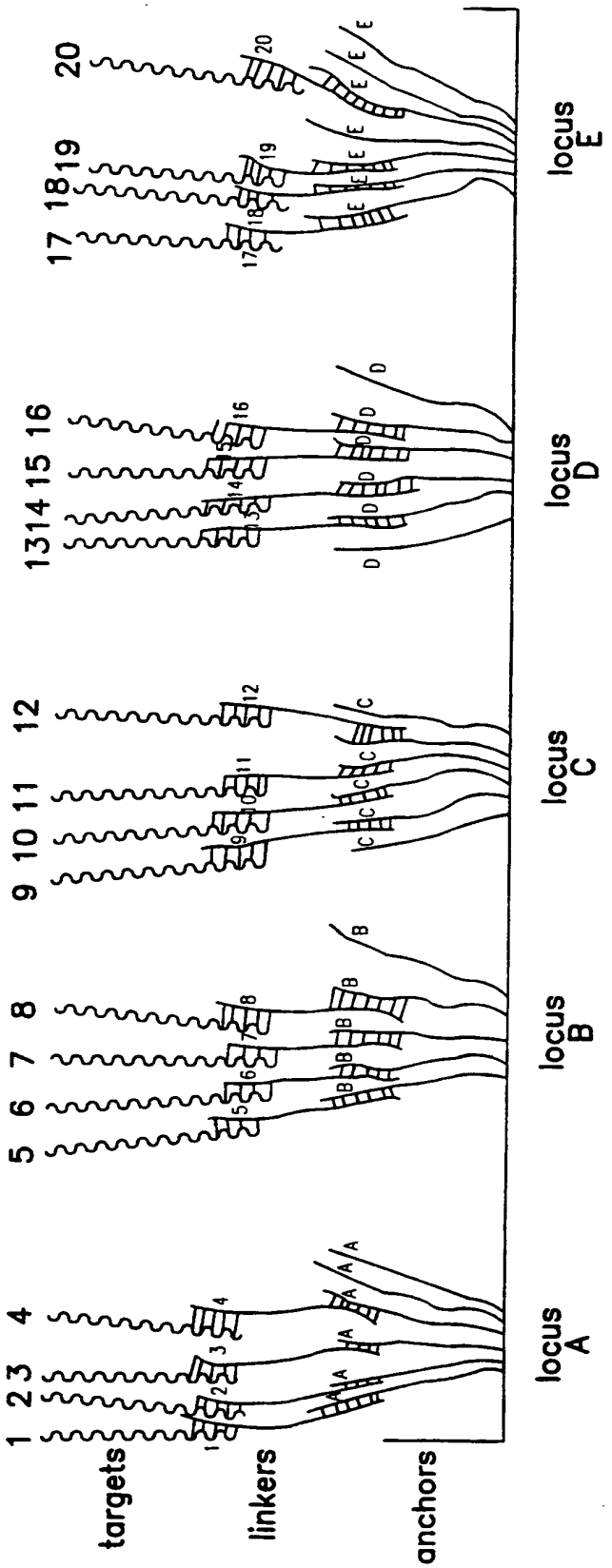


FIG. 25

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 00/16952

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	see page 1 and claim 1	4,14-17, 19,21

Y	WO 97 47640 A (SARNOFF CORP) 18 December 1997 (1997-12-18) see claims	4,15,17, 19,21

Y	US 4 716 106 A (CHISWELL DAVID J) 29 December 1987 (1987-12-29) see whole doc., sep.claims and figures	14,16

X	WO 97 31256 A (BLOK HERMAN ;BARANY GEORGE (US); KEMPE MARIA (US); ZIRVI MONIB (US) 28 August 1997 (1997-08-28) see whole doc. esp. claim 1	1-3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 February 2001	06/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040. Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Mueller, F

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/16952

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	NIEMEYER C M ET AL: "OLIGONUCLEOTIDE-DIRECTED SELF-ASSEMBLY OF PROTEINS: SEMISYNTHETIC DNA-STREPTAVIDIN HYBRID MOLECULES AS CONNECTORS FOR THE GENERATION OF MACROSCOPIC ARRAYS AND THE CONSTRUCTION OF SUPRAMOLECULAR BIOCONJUGATES" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 22, no. 25, 1994, pages 5530-5539, XP000645135 ISSN: 0305-1048 cited in the application see whole doc. esp. figs 3 and 5 and conclusion	1-3
A	EP 0 846 776 A (VYSIS INC) 10 June 1998 (1998-06-10) see figure 3 and p.9, line 26 ff.	1-21
A	US 5 241 060 A (KLINE STANLEY ET AL) 31 August 1993 (1993-08-31) see whole doc. esp. claims	1-21
P,X	WO 99 32663 A (FELDER STEPHEN ;KRIS RICHARD (US)) 1 July 1999 (1999-07-01) the whole document	1-3,16
E	WO 00 37684 A (FELDER STEPHEN ;KRIS RICHARD M (US)) 29 June 2000 (2000-06-29) the whole document	1-21

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 00/16952

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